CSIC

Grupo de Trabajo de ICLAS/CSIC sobre Métodos Complementarios

ICLAS/CSIC Working Group on Complementary Methods

Centro Regional de Salud Pública
Talavera de La Reina
España
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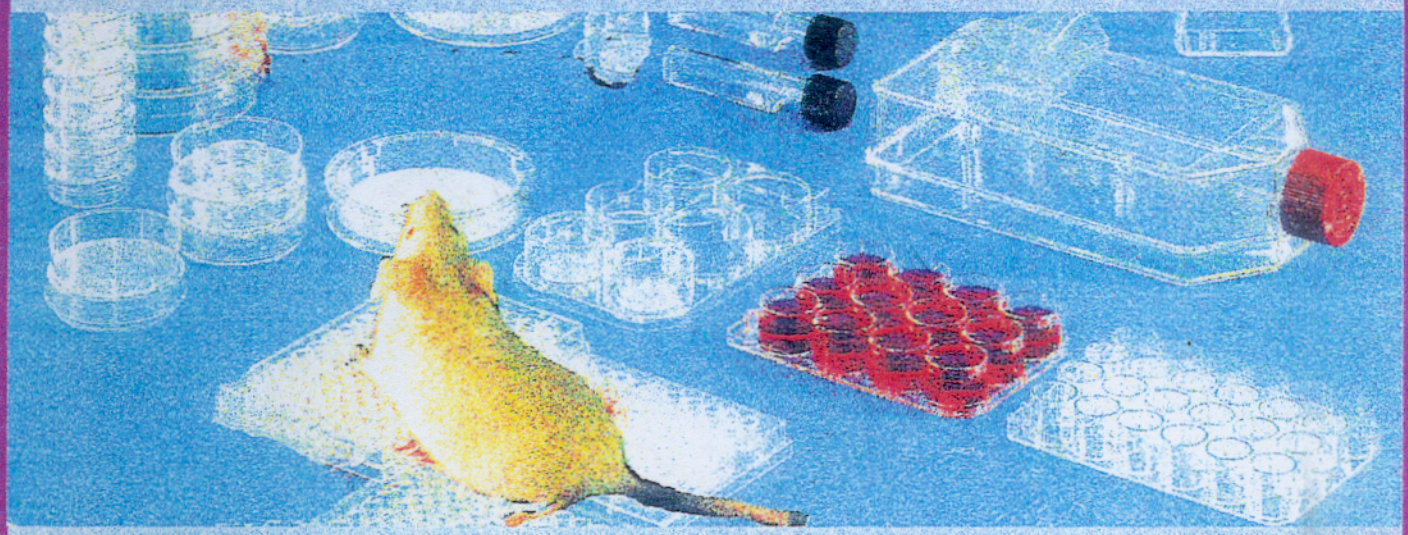

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ICLAS/CSIC Working Group on Complementary Methods

Grupo de Trabajo de ICLAS/CSIC sobre Metodos Complementarios

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FOREWORD

Prof. Jean R. MAISIN

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Consejo Superior de Investigaciones Científicas

Comité Español del ICLAS/CSIC

Centro de Salud Pública. Talavera de la Reina, España.

FOREWORD

The International Council for Laboratory Animal Science was organized in 1956, at the initiative ofthe United Nations Educational, Scientific and Cultural Organization (UNESCO), the Council for International Organization of Medical Science (CIOMS) and the International Union of Biomedical Sciences (IUBS).

ICLAS is an international non-governmental and non-profit-making scientific organization. The aims of ICLAS are to promote international collaboration, quality monitoring and definition of laboratory animals, to collect and disseminate information and to promote the human use of animals in research through recognition of ethical principles and scientific responsibilities.

ICLAS has been stressing the importance of complementary and alter- native methods since a long time and the time has carne to ask the question how far we can go in the use of these methods. Careful planning, refinement of techniques, the rapidly expanding field of biotechnology and the development of non-mammalian models such as monoclonal antibodies and recombinant DNA technology, can lead to the reduction in the number of animals needed to obtain statistically significant answers and to the reduction of animal pain. There is however no chance of replacing all animals in research and testing in the foreseeable future. The search for alternative methods should continue but the hope for a certain success must be tempered by the realization that progress in this area has been slow.

The Governing Board ofICLAS decided in Hyderabad in September 1994 to create a working group on complementary and alternative methods with Dr . de la Peña de Torres as coordinator. The workshop on complementary methods organized in the Centro de Salud Pública Talavera dé la Reina Toledo from 27 through 30 April1995 was the first concrete action of this ICLAS working group.

Several ,well-known scientists participated to this working group which was of a high scientific quality. The following general topics were discussed by the working group: *in vitro* cytotoxicity test; *in vitro* test for carcino- gen/mutagen detection; *in vitro* test for eye/skin irritancy and in vitro test for organ-specific toxicity. I really hope that this workshop will contribute significantly to the reduction of the number of animals used in testing.

I would like to thank very much the organizing committee of this working group and in particular his coordinator Dr. de la Peña de Torres for the work they have achieved.

Prof. J.R. Maisin
President of ICLAS

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Centro Regional de Salud Pública

Talavera de la Reina, España

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ICLAS

ICLAS is a non-governmental organization for international cooperation in laboratory animal science. Its aims are to promote international collaboration, quality monitoring and definition of laboratory animals, to collect and disseminate information and to promote the humane use of animals in research through recognition of ethical principIes and scientific responsabilities.

ICLAS was organized in 1956 at the initiative of UNESCO, CIOMS and IUBS. Since 1961 ICLAS has been officially recognized by the WHO and more recently by ICSU and WVA.

ICLAS has National Scientific, Union, and Associate members. National members represent national authorities and the Scientific members are representatives of different national and regional laboratory animal science associations. Union members represent international non-governmental unions, and the Associate members are commercial and other organizations and institutes which support the aims of ICLAS.

Number of members in December 1994: 8 Honorary, 41 National, 19 Scientific, 8 Union, 68 Associate members.

CSIC

Spain's Scientific Research Council (Consejo Superior de Investigaciones Científicas), best known by its acronym, CSIC, was founded in 1939.

While belonging to the Ministry of Education and Science, the CSIC is at the same time an autonomous body with its own assets and bylaws. It is the most important multidisciplinary research centre in Spain and one of its basic functions is the furtherance of science within its member institutes.

The CSIC has 94 institutes distributed throughout Spain. 39 are in the province of Madrid, 16 in Andalucía, 13 in Cata1onia, 6 in Valencia, 5 in Aragón, 4 in Galicia, 3 in Castilla-León, 2 in the Canary Islands, 2 in Asturias, 1 in Murcia, 1 in the Balearic Islands and 1 in Cantabria. It also has an institute in Rome (Italy). The majority of these research centres belong entirely to the CSIC, but 23 of them are operated on a joint basis with universities.

ORGANIZERS & PARTICIPANTS

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
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Consejo Superior de Investigaciones Científicas

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    CSIC Relaciones Internacionales. Madrid. España.

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17. Dr. Herman B.W.M. Köeter.
    OCDE. Environmental Health and Safety Division. Paris. Francia.

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    AET /INT .Asociación Española de Toxicología. Sevilla. España.

22. Dra. Isabel Rodríguez
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23. Dr. Horst Spielmann
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24. Dras. E. Valcarce

25. A. Herrera y

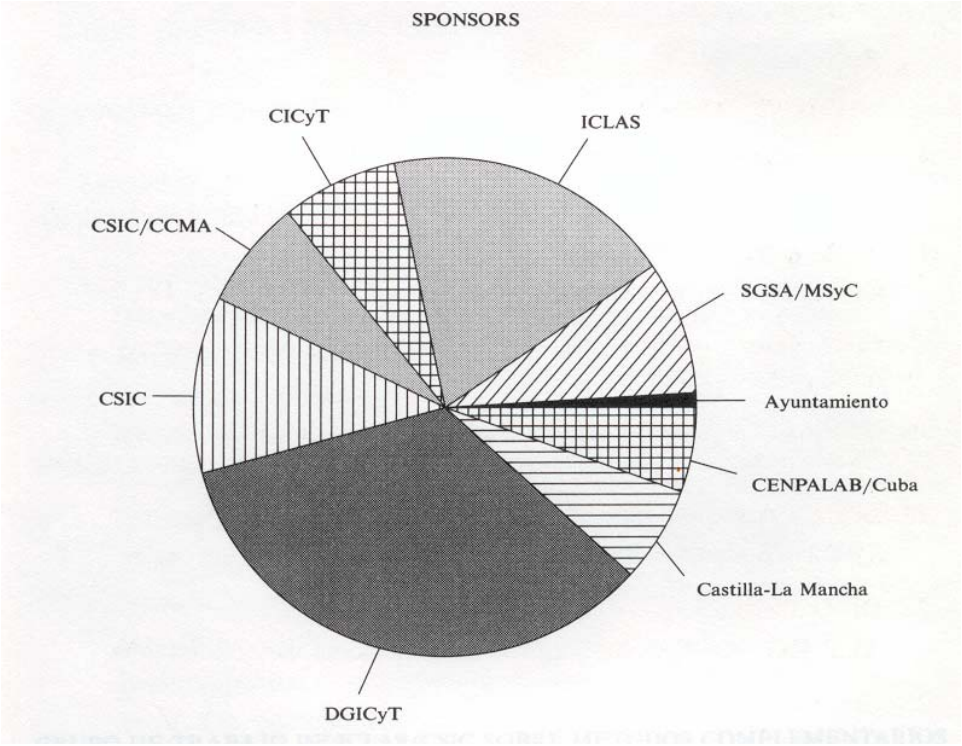
26. C. Caballo.
    MSyC. Ministerio de Sanidad y Consumo. España.

27. Dr. Jorge Zapatero.
    CIDA. SAL. Centro de Investigación y Desarrollo Aplicado, Barcelona España.

28. Srta. Antonia Martínez. Centro de Ciencias Medioambientales. CSIC.

29. D. Stephen Carlin and

30. Dª Ann Gossling.



Entidades Colaboradoras:

- Ayuntamiento de Talavera de la Reina.
- Comunidad de Castilla-La Mancha.
- Centro Regional de Salud Pública de Castilla-La Mancha.
- Centro de Ciencias Medioambientales. CSIC.

- *lnternational Council for Laboratory Animal Science.*
- Consejo Superior de Investigaciones Científicas.
- Secretaría General del Plan Nacional de I+D, CICyT.
- Ministerio de Sanidad y Consumo.
  -Subdirección de Sanidad Ambiental.
- Dirección General de Investigación Científica y Técnica.
- CENPALAB. Centro de Producción de Animales de laboratorio. Bejucal, Habana. Cuba.

ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Dr. Eduardo de la Peña

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
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Town Hall of Talavera de la Reina
The Mayor

Regional Public Health Center
Dr. Juan Atenza

The Community of Castilla-La Mancha
Dr. Rafael Peñalver

International Council of Laboratory Animal Science
Prof. Dr. Maisin represented by Prof. García Partida

Spanish Scientific Research Council
Dr. Pilar Goya

Interministerial Commission for Science and Technology
Prof. Dr. Santiago Lago

Health Ministry
Dr. Francisco Vargas

I would like to extend my thanks to all of those who have accepted our invitation. We will be hearing from important personalities working on the research, spread and validation of methods which are complementary and alternative to animal experimentation (Table I).

TABLE 1

Classification of Alternative Methods
***In vitro* techniques -Embryo culture** -Organ culture and baths -Culture of cell reaggregates -Culture of dispersed cells -Culture of cell lines -Cell free models -Lower organisms: bacteria, algae, etc.
Theoretical prediction models -Relation chemical structure activity (QSAR) -Pharmaco-toxicocynetics (PB- PK)
Teaching models -Mechanical models -Audiovisual systems -Computer simulation and virtual reality

I would like to make special mention of the following speakers:

- Dr. María José GOMEZ LECHON, Hospital de la Fé and author of the book *In Vitro Alternative to Animal Pharma-Toxicology.*
- Prof. Dr. Michael BALLS, Director of ECVAM, European Centre for the Validation of Altemative Methods.
- Dr. Alan GOLDBERG, Director of CAAT, Center for Altematives to Animal Testing located in Baltimore and President of the First World Congress on Altemative Methods held last year in Baltimore, MD (USA).
- Dr. Herman KÖETER, Health and Environmental Security Division of the OECD.
- Prof. Dr. Horst SPIELMANN, Director of ZEBET, National German Center for Documentation and Evaluation of Altemative Methods to Animal Experiments.

A special mention of the scientific members of ICLAS:

- ICLAS Cuban Committee: Dr. Francisco González-Menció.
- ICLAS Finnish Committee: Dr. Kai Pelkonen.

- ICLAS Spanish Committee: Drs. Pilar Goya, Santiago Lago, Francisco Ferrandiz y Paulino Garcia-Partida.

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- From the Health Ministry: Drs. Elina Valcarce, Angustias Herrera, and Covadonga Caballo
- From the Health Institute Carlos III: Dr. Carmen Barrueco and Dr . Bartolomé Ribas
- From CISA in Valdeolmos: Drs. José Vicente Tarazona and Argelia Castaño
- From the Center of Environmental Sciences: Dr. Ana Guadaño and Dr. Azucena González

A special mention of the representatives of Scientific Associations and Societies:

- SEMA, Spanish Mutagenesis Society: Dr. Carmen Pueyo.
- AET, Spanish Toxicology Association: Dr. Guillermo Repetto.
- SEEA, Spanish Animal Experimentation Society: Prof. Paulino García Partida.
- SEQM, Spanish Medical Chernistry Society: Dr. Pilar Goya.
- ISCE, Intemational Society of Chemicals Ecology: Dr. Azucena González Coloma.

A special mention of those representing private control centers:

- From CIDA in Barcelona: Dr. Jorge Zapatero.
- From FIFA/University of Navarra: Dr. Adela López de Ceraín.
- From GAIKER in Zamudio: Dr. Isabel Rodríguez.

A special mention of the secretary members:

Drs. Carmen Barrueco, Ana Guadaño and Azucena González,
Lda. Mercedes García Vedia and Miss Antonia Martínez.

and especially to the director of the Regional Public Health Center of Talavera de la Reina, Dr. Juan Atenza, who has made the facilities of the center available to us.

BACKGROUND OF ICLAS

The opportunity to hold this Working Group, Talavera '95.

ICLAS (International Council on Laboratory Animal Science) has been stressing the importance of complementary and alternative methods to animal experimentation for years now. In the ICLAS Regional Meeting held in Madrid (1982), several presentations were presented on bioassays methods on complementary or alternative methods. In 1983, the ICLAS/CSIC Spanish Committee, in collaboration with the General Councils of Pharmacists, Medical Doctors and Medical Veterinaries, published a list of Ethical Principles in Animal Experimentation (Figure 1). Article 7 and this list makes reference to the use of Complementary / Alternative Methods to Animal Experimentation. In the ICLAS Regional Meeting held in Aguas de Lindoia (Brazil) we made a presentation on complementary methods entitled " Alternatives to animal experiments in the toxicology and ecotoxicology research program" (Laborda, de la Peña *et al.* 1986). More recently, during the last ICLAS Regional Scientific Meeting held in Hyderabad (India), the president of ICLAS, Prof. Maisin, in his inaugural presentation made special mention of the development of complementary methods as well as the important work being done by ECVAM, European Centre for the Validation of Alternative Methods, Environment Institute in the Joint Research Centre of Ispra, Italy (Maisin, 1994).

The importance of the development of new complementary methods led to the creation of the *Working Group on Complementary Methods* during the ICLAS board of directors meeting in Hyderabad. I was made responsible for the coordination of this group and it is my hope that we get a lot accomplished in this working group. The conclusions and agreements reached here were presented in July of this year in the Helsinki Assembly and Congress.

SOCIAL CALL FOR COMPLEMENTARY ASSAYS

Today's society is calling for assays which use less animals or which use no animals at all. This call is the social answer to the three Rs proposed by Rusell and Burch (1959):

1) *Reduction* in the number of animals used.
2) *Refinement* of the assays in which animals are used.
3) *Replacement* of assays which use animals.

VALIDATION OF COMPLEMENTARY METHODS

This validation can be considered from a scientific, political and commercial point of view with regard to evaluation, replacement and other aspects (Balls, 1994).

The validation of assays touches upon three fundamental aspects:

1) Selection of the chemical compounds used in the validation process;
2) Availability of *in vivo* data to be used as a point of reference when assessing the *in vitro* results;
3) The method used to analyze, compare and interpret *in vivo* and *in vitro* data.

ACCEPTANCE OF COMPLEMENTARY METHODS

They must be legally approved before they can be used to test chemical and pharmaceutical substances. This stage of legal acceptance follows intra and inter-laboratory confirmation and the development of a data base which comprises the validation process.

In this area, the 15 Recommendations of the CAAT/ERGATT Workshop on the Validation of Toxicity Test Procedures (Balls et al. 1990) should be considered.

The OECD has drawn up a series of monographs on validation as well as methods guides: Scientific Criteria for Validation on *in vitro* Toxicity Test (OECD 1990), and Guidance Document for the Development of OECD Guidelines for Testing of Chemicals (OECD, 1993).

LEGISLATION

The legislation concerning complementary and alternative methods to the use of laboratory animals (EEC Directive 86/609) makes mention of the use of alternative methods in articles 7 and 23 and in the March 14 Royal Decree 223/1988, on the protection of laboratory animals used for experiments and other scientific purposes (BOE Boletín Oficial del Estado, 18 March, 1988); in the Community of Madrid, the Office of Agriculture and Cooperation emitted an order (4 August, 1989) concerning the protection of animals used for experiments and for other scientific purposes (BOCM Boletín Oficial de la Comunidad de Madrid, 24 August, 1989).

The EEC Directive 93/35 on cosmetics sets the date of January 1998 as the deadline for the use of laboratory animals in the evaluation of the toxicology of their components given that alternative methods exist.

DEVELOPMENT OF THE WORKING GROUP

The ICLAS/CSIC Working Group on Comp1ementary Methods was divided into five work sessions, each of which began with a presentation by an invited speaker. The subjects and speakers were as fo11ows:

- Session I/Speaker Dr. Herman Köeter. *International Harmonization of Test Methods for Hazard Characterisation Taking Into Account Animal We/fare Issues.*
- Session II / Speaker Dr. Horst Spielmann. *The Validation of in vitro Toxicity Test Procedures in Europe.*
- Session III / Speaker Dr. Ma José Gómez Lechón. *In vitro Assays in the Development of Drugs and Chemicals.*
- Session IV / Speaker Dr. A1an Goldberg. *Development of Alternative Methods and the Modular Approach to Validation.*
- Session V / Speaker Dr. Michae1 Bal1s *The Development, Validation and Acceptance of Alternative Methods.*

The presentation made by each one of the speakers was fo11owed by a discussion that was summarized by the rapporteurs and moderators of each session in order to put together a work document. The summaries were agreed upon in session VI.

- Session VI/ Overall Review, Summary and Conc1usions. Secretary, Dr. Gonzá1ez Menció. Rapporteurs, H. Spie1mann, A. Lopez de Ceraín and C. Barrueco.

Conclusions and Clausure

The following is a 1ist of the general aspects to be considered throughout the Working Group on Comp1ementary and/or A1ternative Assays:

1) *In vitro* cytotoxicity test.
2) *In vitro* test for carcinogen/mutagen detection.
3) *In vitro* test for eye/skin irritancy (Draze irritancy test).
4) *In vitro* test for organ-specific toxicity.

[4]

It wou1d 1ike to thank each one of the participants for his/her effort and to the members of the organization committee for their work before and during the meeting.

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

International Council for Laboratory Animal Science

COMITE ESPAÑOL DEL ICLAS/CSIC

PRINCIPIOS ETICOS DE LA EXPERIMENTACION ANIMAL

Principios básicos

Artículo 1. Los progresos del conocimiento humano, son necesarios y sobre todo los de la biología, de la medicina del hombre y de los animales.

Articulo 2. El hombre tiene necesidad de utilizar el animal en la búsqueda del conocimiento humano igual que para alimentarse, vestirse y trabajar. De ahí el deber de respetar el animal, entre auxiliar y ser viviente común a él.

Artículo 3. Toda persona que emplee animales con fines experimentales debe tener presente que están dotados de sensibilidad y memoria y son susceptibles al dolor y sufrimiento.

Responsabilidades del experimentador

Artículo 4. El experimentador es moralmente responsable de sus actos en el marco de la experimentación animal.

Artículo 5. Las experiencias concernientes a los seres vivos y las extracciones de tejidos a sujetos vivos con fines de investigación deben ser realizados por un científico cualificado o bajo su control directo. Las condiciones de conservación de los animales en experimentación deben ser definidas y controladas por un veterinario o por un científico competente.

Artículo 6. En los estudios sobre la utilización de animales debe existir una probabilidad razonable para que estos estudios contribuyan de manera importante a la adquisición de conocimientos que desembocan eventualmente en la mejora de la salud y del bien- estar del hombre y de los animales.

Artículo 7. Los métodos estadísticos, los modelos matemáticos y los sistemas biológicos *in vitro* deben ser utilizados cuando sean apropiados para completar la experimentación animal y para reducir el número de los sujetos utilizados.

Artículo 8. El experimentador debe utilizar el animal mejor adaptado a su investigación y tener en cuenta también los grados sensoriales y psíquicos propios de cada especie. Los animales en peligro de extinción no deberán ser utilizados más que en circunstancias excepcionales muy definidas. Mientras sea posible, los animales utilizados en el laboratorio provendrán de crías especializadas para asegurar las mejores condiciones de equilibrio biológico.

Artículo 9. El experimentador debe velar porque las condiciones de conservación del animal de laboratorio sean las mejores posibles y aportar los cuidados necesarios antes, durante y después de las intervenciones.

Artículo 10. El experimentador tiene el deber de ahorrar al animal todo sufrimiento fisico o psíquico inútil. Debe poner en marcha los métodos que permitan limitar el sufrimiento y los dolores en el caso o casos que sean inevitables.

Figure 1

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INTERNATIONAL HARMONIZATION OF TEST METHODS FOR HAZARD CHARACTERIZATION TAKING INTO ACCOUNT ANIMAL WELFARE ISSUES

Dr. Herman B.W.M. Köeter

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Consejo Superior de Investigaciones Científicas

Comité Español del ICLAS/CSIC

Centro de Salud Pública. Talavera de la Reina, España.

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Session I/Sesión I

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INTERNATIONAL HARMONIZATION OF TEST METHODS FOR HAZARD CHARACTERISATION TAKING INTO ACCOUNT ANIMAL WELFARE ISSUES

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INTRODUCTION

Today, some 100,000 chemicals are in commercial use. Fifteen hundred of these chemicals are considered as High Production Volume (HPV) chemicals with a yearly production of more than 10,000 tons each. Of these HPV chemicals, some 500 are identified for which little or no data is available in the open literature. In order to decrease the number of (potentially) hazardous compounds on the market, regulation of new chemical entities generally involves a substantial number of toxicity tests. In addition, the OECD countries decided in 1987 to undertake a systematic investigation of existing chemicals. Both activities include toxicity testing.

HAZARD IDENTIFICATION: SOME GENERAL CONSIDERATIONS

The most fundamental question, being the comerstone of chemical management, is: *what are acceptable risks of chemical exposure?* In order to be able to answer this question, one must first identify the potential hazards and classical tools of the hazard identification process still are animal studies.

Principles that are considered most relevant in toxicity testing in general are:

- Identification of the *nature* of the toxic effect (qualitative approach);
- Assessment of the *dose* needed to induce the toxic effect (quantitative approach);
- Understanding the *mechanism* underlying the toxic effect (mechanistic approach).

[1] The opinions presented in this paper do not necessarily represent the opinions of the OECD or its Member countries and should therefore be viewed as those of the author.

In general, animal studies required for regulatory purposes only address the first two aspects. Although this information indeed is of crucial importance as the basis for human safety evaluation (Hart and Fishbein, 1985), it is in many ways deficient. The toxicologica1 expression of a chemical insu1t may vary between species including man or be species-specific. Next, the dose needed to induce an adverse effect mostly relates to dosages given to the animals rather than concentration at the site of action (target organ). As a consequence, uncertainty factors being a clear expression of the inability of the toxicologist to scientifically determine the human hazard of a defined exposure, are still necessary in hazard assessment procedures. Consequently, information about the underlying mechanism of toxic action, including knowledge of the metabolic fate of the chemical substance in the animal species tested, would reduce or occasionally even eliminate these uncertainty factors.

Today a variety of animal studies is available covering acute, repeated-dose and long-term toxicity, carcinogenicity, neurotoxicity, immunotoxicity and genetic and reproductive toxicity. An overview of all currently available OECD Test Guidelines in the area of human effects assessment is presented in Table 1.

TABLE 1: ADOPTED OECD TEST GUIDELINES ON HEALTH EFFECTS

No	Title	Date of Adoption
401	Acute Oral Toxicity	24 February 1987
402	Acute Dermal Toxicity	24 February 1987
403	Acute Inhalation Toxicity	12 May 1981
404	Acute Dermal Irritation/Corrosion	17 July 1992
405	Acute Eye Irritation/Corrosion	24 February 1987
406	Skin Sensitisation	17 July 1992
407	Repeated Dose 28-day Oral Toxicity in Rodents	27 July 1995
408	Subchronic Oral Toxicity -Rodent: 90-Day	12 May 1981
409	Subchronic Oral Toxicity -Non-Rodent: 90-Day	12 May 1981
410	Repeated Dose Dermal Toxicity: 21/28-Day	12 May 1981
411	Subchronic Dermal Toxicity: 90-Day	12 May 1981
412	Repeated Dose Inhalation Toxicity: 28/14-Day	12 May 1981
413	Subchronic Inhalation Toxicity: 90-Day	12 May 1981
414	Teratogenicity	12 May 1981
415	One-Generation Reproduction Toxicity	26 May 1983
416	Two-Generation Reproduction Toxicity	26 May 1983
417	Toxicokinetics	4 April 1984
418	Delayed Neurotoxicity of Organophosphorus Substances following Acute Exposure	27 July 1995

No.	Title	Date of Adoption
419	De1ayed Neurotoxicity of Organophosphorus Substances: 2º8-day. Repeated Dose Study	27 July 1995
420	Acute Oral Toxicity -Fixed Dose Method	17 July 1992
421	Reproduction/Developmental Toxicity Screening Test	27 July 1995
451	Carcinogenicity Studies	12 May 1981
452	Chronic Toxicity Studies	12 May 1981
453	Combined Chronic Toxicity/Carcinogenicity Studies	12 May 1981
471	Genetic Toxicology: *Salmonella typhimurium,* Reverse Mutation Assay	26 May 1983
472	Genetic Toxicology: *Escherichia coli,* Reverse Mutation Assay	26 May 1983
473	Genetic Toxicology: *In vitro* Mammalian Cytogenetic Test	26 May 1983
474	Genetic Toxicology: Micronucleus Test	26 May 1983
475	Genetic Toxicology: *In vivo* Mammalian Bone Marrow Cytogenetic Test -Chromosomal Analysis	4 April 1984
476	Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests	4 April 1984
477	Genetic Toxicology: Sex-Linked Recessive Lethal Test in *Drosophila melanogaster* .	4 April 1984
478	Genetic Toxicology: Rodent Dominant Lethal Test	4 April 1984
479	Genetic Toxicology: *In vitro* Sister Chromatid Exchange Assay in Mammalian Cells	6 October 1986
480	Genetic Toxicology: *Saccharomyces cerevisiae,* Gene Mutation Assay	6 October 1986
481	Genetic Toxicology: *Saccharomyces cerevisiae,* Mitotic Recombination Assay	6 October 1986
482	Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro*	6 October 1986
483	Genetic Toxicology: Mammalian Germ Cell Cytogenetic Assay	6 October 1986
484	Genetic Toxicology: Mouse Spot Test	6 October 1986
485	Genetic Toxicology: Mouse Heritable Translocation Assay	6 October 1986

OECD TEST GUIDELINES PROGRAMME

The OECD Test Guidelines are considered the leading international standard for safety testing and, indeed, are an important activity of the OECD. Consequently, alternative test methods, either using smaller numbers of animals, or using *in vitro* techniques, once adopted by OECD, would carry considerable weight and lead to the fervidly desired international acceptance. In order to increase the awareness of the possibilities that exist to give input to the Test Guidelines Programme and, in particular, to support the development of alternative *(in vitro)* test methods, a full understanding of the programme is essential. Although the OECD Test Guidelines as such are well-known, it is certainly less known how these guidelines are developed. To this end, a monograph has been drafted, explaining the structure of the Test Guidelines Programme, the various levels of responsibility and ways of giving input to the Programme (OECD, 1993a).

An essential aspect of the OECD is that this organisation is not a supranational organisation but rather a center for discussion where governments express their points of view, share their experiences and search for common ground. This implies that decisions are made by consensus. Once the Council, which is the highest authority of the OECD, adopts a formal Decision such a decision is binding on all Member countries. The OECD Guidelines for the Testing of Chemicals (OECD, 1993b) form an integrated part of such a binding Council Decision (OECD, 1981). The other subject of this Council Decision, which is less known is that on Mutual Acceptance of Data. In the Council Decision it is stated that: "Data generated in the testing of chemicals in an OECD Member country in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice (GLP) shall be accepted in other Member countries for purposes of assessment and other use relating to the protection of man and the environment". In cases where this OECD Decision has not been complied with by an OECD Member country, a specific form could be used to inform the OECD Secretariat of such a case (OECD Notification of Incomplete Implementation of the OECD Decision on Mutual Acceptance of Data). The information provided will be kept confidential and the Environmental Health and Safety Division of the OECD Secretariat will conduct an investigation of the complaint in the Member country involved.

The process of Test Guideline development comprises two parts. First, the need for a guideline is to be defined. The various steps of this part of the process are indicated in Figure 1.

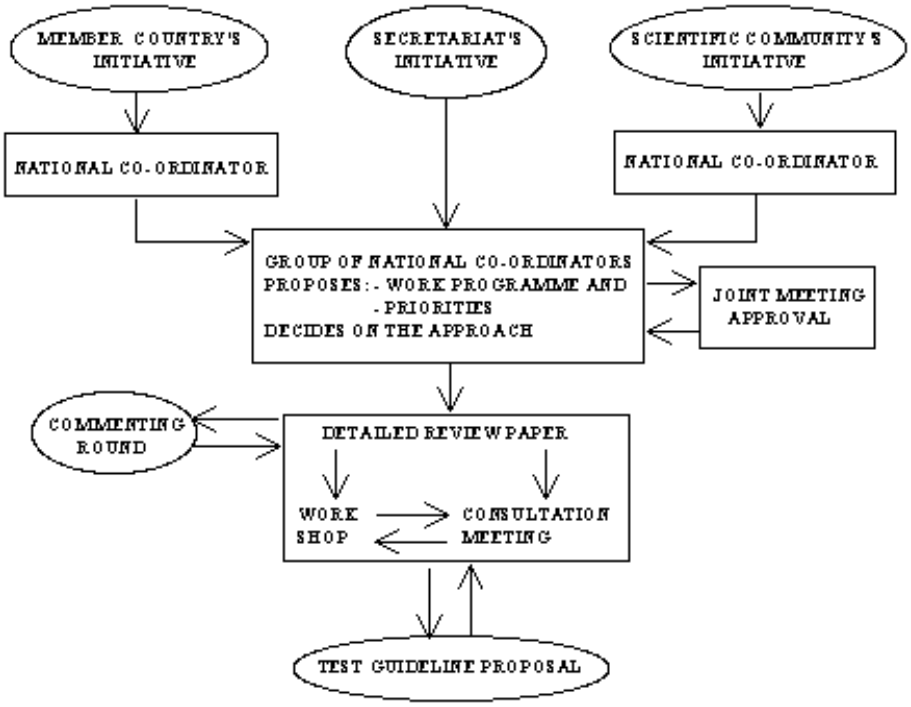

Figure 1: OECD Test Guideline development; Step 1: defining the need.

TABLE 2. Criteria for considering a test guideline proposal for development as OECD Test Guideline.

- The proposed test should properly address the end-points concerned,
- in addition, the proposed test should have undergone a critical appraisal concerning its:
  - scientific justification,
  - sensitivity,
  - reproducibility,

  including, where feasible and relevant, a comparative study, supporting the validity of the test proposed,
- further, the test should allow for standardization, and
- not normally require unique equipment or technical experience.

As clearly indicated in this diagram, the National Co-ordinators of the Test Guidelines Programme play an important role in this process. The initiative to start the development of a particular guideline can be taken by the OECD Secretariat, by one or more Member countries or, most importantly, by the scientific community itself. Proposals, received by the Secretariat are discussed at the annual Meeting of National Co-ordinators. During these meetings, priorities for future activities are set and the approach that should be followed in dealing with the selected activities is discussed. Quite often, SO-called Detailed Review Papers (DRPs) form the basis of a new or updated guideline. These DRPs, which are either prepared by a Member country or by a consultant appointed by the Secretariat, describe the current state-of-the- art in scientific progress and technical possibilities of a well-defined area of research.

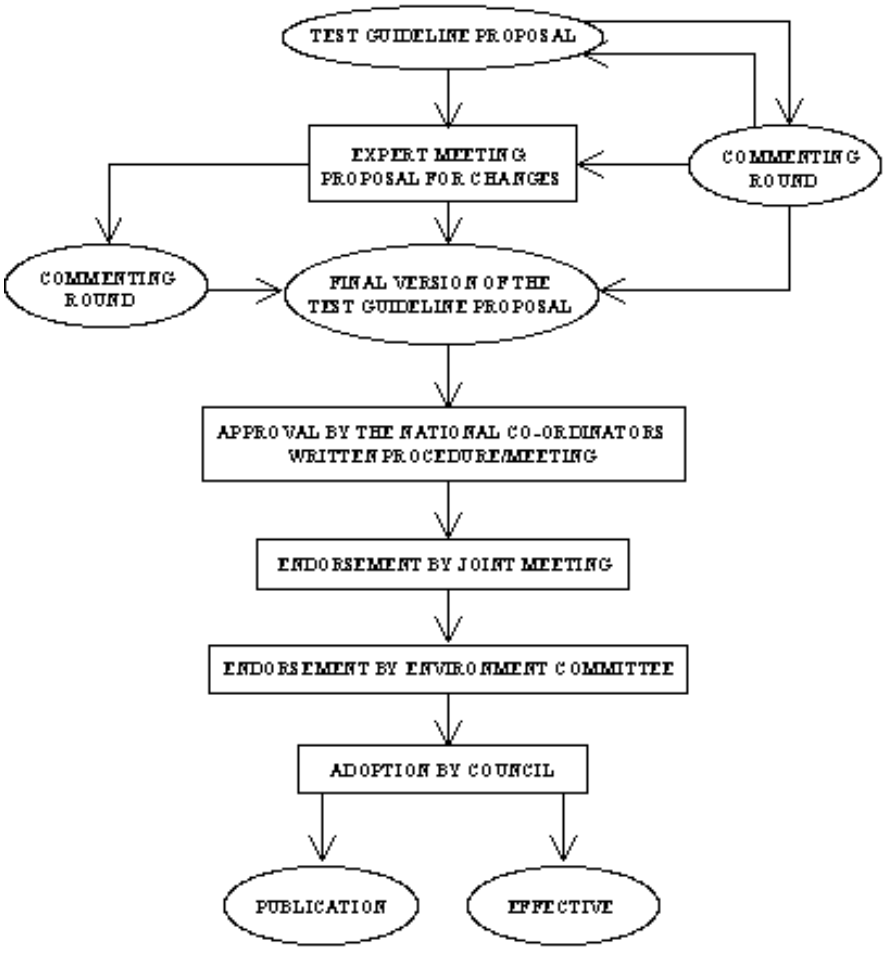

Figure 2. OECD Test Guideline development; Step 2: producing the product.

After completion, either an expert meeting or a commenting round will be organised. All Member countries will have sufficient opportunities to express their views. When the DRP is acceptable to the experts of all Member countries, the second part is the actual development of a Test Guideline, based on recommendations as included in the DRP. When the need to develop or update a Test Guideline is clear-cut, the OECD Secretariat may arrange for the preparation of a draft Test Guideline proposal without a DRP. The criteria that apply in order to consider a proposal for development as OECD Test Guideline are presented in Table 2.

Not surprisingly, the most relevant criterion, being the validity of the test proposed, is, at the same time, the most difficult one to demonstrate. The part of the process that describes the procedure of actual Test Guideline development is indicated in Figure 2. Similar to the procedure followed for the DRP, the Test Guideline proposal will be circulated for comment to relevant experts in all Member countries nominated by their National Co-ordinator. Frequently, in addition to the commenting rounds, Test Guideline proposals are discussed in special expert meetings. Once the experts reach consensus on a particular Test Guideline, the proposal is put forward to the Meeting of the National Coordinators for approval. Since each Guideline will form an integrated part of the earlier mentioned Council Decision, each Guideline also needs formal adoption by the Council before it becomes effective.

OECD AND ANIMAL WELFARE

With respect to animal welfare, the OECD has taken a rather pragmatic position. At the second High Level Meeting in 1982, bringing together ministers and other high level officials, the following statement was adopted: " *The welfare of laboratory animals is an important factor influencing the work in the OECD Chemicals Programme. The progress in OECD on the harmonization of chemicals control, in particular the agreement on mutual acceptance of data, by reducing duplicative testing, will do much to reduce the number of animals used in testing. Such testing cannot be eliminated at present, but every effort should be made to discover, develop and validate alternative testing systems"* (emphasis added). In 1987, when some existing guidelines on health effects were updated, animal welfare was addressed indeed. As a result the number of animals required for acute toxicity testing was reduced and the probability of severe animal suffering was diminished (Updated Test Guidelines 401, 402 and 405). At the same time, the updated Test Guideline on Acute Eye Irritation/Corrosion (Test Guideline 405) provided for the use of *well-validated* alternative studies to identify corrosive or severe irritating sub-

stances. Now, substances recognized as such in the alternative test need not be further tested for eye irritation, it being presumed that such substances will produce similarly severe effects on the eyes in a live animal test. Because the High Level Meeting of 1982 had recommended that alternative methods be validated before they can be applied in hazard identification schemes, there was a need to define the scientific criteria for validation of alternative methods. In a document on this subject prepared for the OECD, Frazier (1990) describes a three-step approach consisting of: micro (intralaboratory) validation, macro (interlaboratory) validation, and test battery optimization (given several test systems, compose the most relevant combination). In the report of the CAAT *IERGATT* Workshop on the validation of toxicity test procedures more or less the same strategy was recommended (Balls et al, 1990). CAAT is The Johns Hopkins Center for Alternatives to Animal Testing and ERGATT is the European Research Group on Altematives to Animal Testing. However, this Workshop expanded the validation scheme with a "test data base development phase". Further, they made clear recommendations with respect to the numbers of reference chemicals that should be tested at each phase, in total amounting to as many as 235-330 compounds. Recently, again under the auspices of CAAT and ERGATT , a follow-up workshop on the issue of validation concluded that the criteria for validation should be more flexible. (Balls *et. al.,* 1995).

Most papers that deal with the issue of test validation focus on the need to show reproducibility and high correlations with the existing animal test they want to replace. The higher the number of chemicals tested and the bigger the number of participating laboratories, the better the validation project. From a statistical viewpoint this may certainly be true. However, not only is such a validation process very time-consurning and extremely expensive, it also lacks the fundamental basis of proving mechanistic similarity to the phenomenon we want to study. In other words, when one wants to assess a particular hazard (either human or environmental), the question should be asked: does the alternative test give sufficient information to be able to make such an assessment? Reasoning along these lines, Flint (1992) even suggested applying only one rule for validation, namely: " *Al[tests must have demonstrable mechanistic similarity to the events we wish to study in vivo".* Although this approach rightly puts necessary emphasis on the importance of the fundamental scientific principle of acceptance of alternative lÍ1ethods by understanding the mechanisms involved, it will nevertheless also remain necessary to prove the reproducibility of a promising method.

In order to avoid confusing the issue and cause further delay, the philosophy of *learning by doing* could be adopted. This rather pragmatic approach has

proved to be successful in the OECD Existing Chemicals Programme (Brydon et al, 1990). In this context it would mean that incorporating "alternative" test methods in safety testing programmes, not to the exclusion but in addition to animal studies, even when they are not (fully) validated, will offer the possibility of a better understanding of the potential and limitations of the selected alternative under various test conditions. At the same time a valuable data base could be developed. Such parallel or tiered testing may appear to be a practical and more realistic way of demonstrating the quality (the intrinsic value, sensitivity, reproducibility, and selectivity) of se1ected alternative tests.
Consequently, in the updated version of the Test Guideline 404 on Acute Dermal Irritation/Corrosion, and Test Guideline 406 on Skin Sensitisation, adopted by the OECD Council in July, 1992, there is now room for alternative methods that are not (yet) formally validated. In Test Guideline 404 it is stated that "it may not be necessary to test *in vivo* materials for which corrosive properties are predicted on the basis of results from *in vitro* tests". Similarly, Test Guideline 406 allows the use of initial, less-invasive animal screens to detect sensitizing substances. However, as *conditio sine qua non* each alternative test should be conducted fully in compliance with the Principles of Good Laboratory Practice (GLP) and be similarly reported as a full animal study.

Regulatory Acceptance of Alternative *(In Vitro)* Tests

A scenario for the development of *in vitro* tests that could be followed with a reasonable chance of international regulatory acceptance, is summarized in Table 3. The approach consists of a number of subsequent steps. Most importantly, it should be understood that it is of crucial importance to recognize and develop simple, differentiated endpoints, essential for hazard identification. For instance, an *in vitro* assay should address a clearly defined endpoint rather than covering in one test all the phenomena of the *in vivo* endpoints. Complex processes which are already difficult to understand in *in vivo* studies, can never be transposed as such to *in vitro* studies. It should be realized that considerable numbers of *in vitro* assays are necessary to fully cover a particular *in vivo* event. Next, during the process of developing an *in vitro* method, the mechanism of the test should be understood and the significance of its endpoints as indicators of adverse effects on the actual target event should be considered. This implies that the in vitro test should not in the first place seek high correlations with an existing *in vivo* assay, but mimic as close as possible a particular aspect of the real event for which the hazard should be assessed. Having accomplished this, the method should be submitted for publication. Only acceptance of the paper by a high quality, peer reviewed toxicology jour-

nal could be considered as the scientific justification of the test. This strong foundation is essential for further development and international acceptance of the test.

In order to develop a sound database, the *in vitro* assay(s) initially should be conducted parallel to the relevant *in vivo* studies as an integrated part of the set of safety evaluation studies, as required by regulatory authorities. In this respect, an important role could be played by industry and contract laboratories, where extensive experience is available with safety evaluation studies, conducted under various conditions, by various dose routes and in various species. In addition, full knowledge of the toxicity profile of a compound under study is almost indispensable for a meaningful interpretation of an observed effect, be it an *in vivo* or *in vitro* study. A crucial step of this scenario for *in vitro* test development is that the alternative tests be reported, interpreted and subsequently integrated in the notification dossier that is submitted to regulatory authorities. Consequently, like all *in vivo* studies, all alternative tests should be conducted and reported fully in compliance with GLP.

A database developed as described above does not necessarily need to be of a particular size, set in advance. By scientific judgement, it could be decided when sufficient data has been collected to demonstrate that the *in vitro* assay indeed covers a particular event for which the hazard of chemical exposure needs to be identified, \hat test results are reproducible, and that the assay has proven to be sufficiently sensitive under various conditions. As the final step of the scenario, the alternative test(s) should be proposed to the OECD to be considered for Test Guideline development, which would be the best possible means to achieve international acceptance.

TABLE 3. Approach for the development of alternative *(in vitro)* test methods that could possibly lead to regulatory acceptance

SCIENTIFIC JUSTIFICATION	1. Select simple endpoints essential for hazard identification. 2. Develop *in vitro* assays for these endpoints. 3. Understand the mechanism of the *in vitro* assay and demonstrate similarities to the target event. 4. Publish the assay(s) in a high quality peer-reviewed journal.
DATABASE DEVELOPMENT	5. Conduct the *in vitro* assays parallel to the relevant *in vivo* studies. 6. Conduct and report all studies fully in compliance with GLP. 7. Integrate results of the *in vitro* assays in dossiers submitted to regulatory agencies.
INTERNATIONAL ACCEPTANCE	8. Propose the *in vitro* assay(s) to the OECD to be considered for Test Guideline Development.

PERSPECTIVES

In the short run, the acceptance of alternative tests may well lead to a minor reduction of animals in existing *in vitro* studies and may even lead to the "extinction" of some of the most objectionable animal tests. However, the policy of replacing existing live animal studies by alternative methods will most probably never lead to a dramatic reduction of the number of animal used for hazard identification purposes. This seemingly pessimistic viewpoint may be explained by using the following metaphor (Köeter, 1991). The most complete set of toxicity data on the basis of which hazard assessment is made, could be compared with the picture of a completed jigsaw puzzle. Each piece of this jigsaw represents a particular study, and the picture informs us about the hazard. To keep the picture complete either all original pieces of the jigsaw should be collected and fit together or worn-out, lost or discarded pieces should be replaced by new ones (alternative tests) that should fit exactly. This very time consuming process will at best result in exactly the same picture that we had before. In other words, no scientific progress as far as hazard assessment is concerned. A better approach, however, would be to keep the old jigsaw, preferably patched up by replacing the most worn-out pieces and, at the same time, create a completely new jigsaw. This new puzzle, when completed, will show a different and probably nicer picture. Moreover, the fine details of this new picture may well be of a better quality than those of the old puzzle. As a result, the new picture could very well be much better scientific basis for future hazard assessment of chemicals. It should be clear, however, that toxicological research, aimed at the development of such new endpoints as part of the future set of toxicity data will differ from research aimed solely at the substitution of a particular test. First, there is no need to cover exactly the same endpoints as measured *in vivo;* second, the research could be focused on understanding of mechanisms involved, rather than on evaluating symptoms only. Consequently, future hazard assessment will be based not primarily on descriptive science, but principally on the understanding of mechanisms at the molecular, cellular and tissue level.

Simultaneously with this long term policy, the development of alternatives to existing tests and the subsequent validation of these new tests should be continued because it will take considerable time before the old jigsaw puzzle can be thrown away. And it certainly needs some repair in order to allow its further use!

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THE VALIDATION OF IN VITRO TOXICITY TEST PROCEDURES IN EUROPE

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GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
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ICLAS/CSIC WORKING GROUP ON
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Session II/Sesión I I

THE VALIDATION OF IN VITRO TOXICITY TEST PROCEDURES IN EUROPE

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The Validation of *In Vitro* Toxicity Test Procedures in Europe

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Abbreviations

ZEBET = Zentralstelle zur Erfassung und Bewertung von Ersatzund Erganzungsmethoden zum Tierversuch.

= National German Center for Documentation and. Evaluation of Alternative Methods to Animal Experiments.

BgVV = Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinarmedizin.

= Federal Institut for Health Protection of Consumers and Veterinary Medicine.

SUMMARY

In 1989 the "Center for Documentation and Evaluation of Alternative Methods to Animal Experiments" (ZEBET) was established at the Institute of Veterinary Medicine of the Federal Health Office (BGA) in Berlin. ZEBET is not only responsible for the documentation of alternative methods but also for validation and acceptance of the new methods at the national and internationallevel, e.g. by the EU or OECD.

In 1992 the European Centre for Va1idation of Altemative methods (ECVAM) was established at the European Joint Research Centre (JRC) in Ispra (Italy). ECVAM is promoting and funding validation activities at the European level. ECVAM has set standards for the formal validation process and ECVAM tries to harmonise them in cooperation with intenational scientific and regulatory bodies. In close cooperation ZEBET and ECVAM have managed and participated in several European validation studies on altematives to the Draize rabbit's eye test and on *in vitro* testing procedures for phototoxicity and corrosivity. At the national level ZEBET promotes the replacement of a fish test for waste water by a fish cell cytotoxicity test. Both ECVAM and ZEBET are actively promoting the standardization of biostatistics to be used in development, validation and evaluation of *in vitro* test procedures.

INTRODUCTION

At the international level there has been growing concern about the suffering of animals in toxicological testing for regulatory purposes. The first step to reduce animal numbers in regulatory testing was intenational standardization of animal test procedures in toxicology by the OECD in 1981 (OECD, 1981) and an agreement of mutual acceptance of studies carried out according to these guidelines by OECD members states including the European Union (EU). The next consequent step in reducing the use of animals in toxicological testing is the replacement of these tests by *in vitro* test procedures, which have to prove to be valid by undergoing a rigid and formal validation process. During the past decade in Europe scientists in academia and industry as well as regulators have agreed upon a formal validation process which is both time consuming and expensive. Therefore, only the most promising in vitro assays could be validated in intemational ring trials so far. The general approach of validation will be outlined in the present report and a few characteristic examples will be given.

RESULTS

Guidelines for the validation of toxicity test procedures

Since scientific validation of toxicity test procedures had not adequately been addressed in 1990 European and American scientists held a joint workshop and agreed upon recommendations for the formal validation of toxicity test procedures (Balls et al., 1990). These recommendations have been widely accepted and, whenever possible, in Europe validation trials were conducted accordingly. Taking into account the experience gained in several European

validation trials the new European Validation Centre ECVAM sponsored in 1994 a workshop on practica1 aspects of the va1idation of toxicity test procedures to reconsider the theoretical basis for validation (Balls et al., 1995). Since in several of the expensive and time consuming validation studies tests had been included, which had not sufficiently been standardized, it was recommended to put more emphasis on prevalidation before starting formal validation. This was in particular addressed by an ECVAM Task Force on prevalidation which has defined the role of prevalidation in the development, validation and acceptance of altemative methods (Curren et al., 1995). According to this new concept during prevalidation a standard protocol has to be worked out for a newly developed test which then has to be independently tested and approved by another laboratory before the assay should undergo a formal validation under b1ind conditions in several laboratories.

Validation trials of *in vitro* alternatives to the Draize rabbit's eye test

German study on the HET -CAM test and a cytotoxicity assay

Since 1988 in Germany a national validation study on two altematives to the Draize eye test has been conducted under blind conditions in 14 laboratories. It was funded by the German Department of Education and Research (BMBF) and coordinated by ZEBET. To identify severely eye irritating chemicals *(EU labelling R-41)* an empirically developed scoring system was used both for the HET -CAM assay and the IC-50 value for the 3T3 cel1 cytotoxicity test. As previously reported for a total of 200 test chemicals, cytotoxicity data did not sufficiently correlate with *in vivo* Draize eye test data and the sensitiv- ity and specificity of the HET -CAM assay were not coI:tvincing for identifying severely eye irritating chemicals (Spielmann et al., 1991, 1993). Toxicologi- cal endpoints and scoring systems of the HET -CAM assay had been derived empirically. It was, therefore, investigated if modem biostatistical methods facilitate to better identify the most predictive endpoints and/or scores of this particular assay, as e.g discriminant analysis, and to calculate *in vitro/in* vivo correlations, e.g. complex regression methods.

Discriminant analysis revealed that among the 9 endpoints of the HET -CAM assay, coagulation was the only acceptable endpoint for identifying severely irritating chemicals (R-4l). The data also allowed to develop an *in vitro* testing strategy for identifying severely eye irritating chemicals (R-41), which is based on combining coagulation data from the HET -CAM assay with cytotoxicity data and which provides an acceptable sensitivity, predictivity and percentage of false positive results (Spielmann *et al.*, 1995). Complex re-

gression methods gave a better description of the *in vitro/in vivo* correlation between the different endpoints evaluated in the Draize eye test *in vivo* than the usual simple linear models.

EU study validation ofthe BCOP assay

In 1991/93 the DG XI of the EU funded a blind validation trial of the bovine coreneal opacity and permeability (BCOP) assay in 13 European laboratories including ZEBET. In the study the predictive value of the BCOP assay was tested for classifying industrial chernicals according EU classification as eye irritants (R 36) and severe eye irritants (R 41). All chernicals were tested *in vivo* in a contract laboratory in the Draize eye test and assesed using the MMAS score. Comparison of *in vivo* and *in vitro* data revealed that the BCOP assay predicted correctly whether a compound is irritating or nonirritating for 44 of the 52 chernicals (85%) and that sensitivity and specificity of the assay were greater than 84% (Gautheron, 1994). Therefore, the BCOP proved to be quite useful for screening chemicals for their ocular irritation potential.

Worldwide ECIHome Office validation study

In 1992 the DG XI of the EU in cooperation with the British Home Office has initiated an international validation study on the most promising alternatives to the Draize eye test. The principal goal of the study is to provide scientifically credible data for proposing to regulatory authorities that one or more non-whole-animal methods should be adopted as a replacement for the Draize rabbit eye irritation test, and that four target steps related to this goal were to determine whether or not the data obtained in the study indicate that it would be possible:

1) to replace the Draize eye test for identifying severely irritating materials or
2) to replace the Draize eye test for identifying severely irritating materials belonging to specific chemical classes or
3) to replace the Draize eye test completely (i.e. to identify all levels of irritancy of materials without regard to their chemical class) or
4) to replace the Draize eye test for identifying all levels of irritancy of materials belonging to specific chemical classes.

The EC/HO validation exercise is covering 9 non-animal methods established in laboratories of the European chemical industry including organotypic

models and cellular and physicochemical assays. Each test was performed in at least 4 different laboratories in Europe, Japan and the USA and 60 coded chemicals were testes under blind conditions. The study was carried out according to the recommendation of the CAAT /ERGATT workshop on the validation of toxicity test procedures (Balls *et al.* 1990). 60 test chemicals with high quality *in vivo* data covering the whole range of eye irritation between MMAS 0 and 110 had been carefully selected by experts of ECETOC, the European chemical manufacturer's association. Coding and shipping of test chemicals as well as handling and analysis of the data were carried out independently by a contract laboratory as suggested by the first joint US and European workshop on validation (Balls *et al.*, 1990). Due to their experience in validation studies both ECVAM and ZEBET were involved in managing this project. The experiments of this validation trial were finished early in 1994 and the results will be published in 1995. Preliminary evaluation of the results of this study suggests that even the best *in vitro* assays presently available hold promise only for testing surfactants but not for a more wide spectrum of chemicals.

COLIPA validation study ofsurfactants

COLIPA, the European cosmetic, toiletry and perfumery association, has in 1994 started a validation study of *in vitro* assays. Taking into account both the interest of COLIPA member companies and the preliminary evalidation of the outcome of the EC/HO Draize eye test alternaties validation trial, this study is limited to surfactants as far as test chemicals are concerned. The COLIPA surfactants validation trial is carried out under blind conditions and follows the general recommendations of the ECVAM Workshop on validation (Balls et al., 1995). Participants include cosmetic companies from Europe, Japan and the USA. More information about the study has not yet been released and results are expected to be published in 1996.

Validation of *in vitro* Phototoxicity Tests

Acute photoirritation testing is an area, in which *in vitro* models using human or animal tissue seem to be more promising than the animal models developed so far. In 1992 the DG XI of the EU and COLIPA have started a joint validation project of acute *in vitro* phototoxicity tests, which was initiated and managed by ZEBET with 8 laboratories participating in 4 European countries. The goal of the project was to determine if currently selected *in vitro* methods are capable of properly predicting the photo-irritation potential to

humans of chemicals applied via the systemic route or topically to the skin. The results are summarised in *Table* 1.

TABLE 1. Summarised data of phase I of the EU/COLIPA *in vitro* phototoxicity validation study

	Mechanistic Assays			Commercial Assays		Growth Inhibition Assays			
	Histidine Photo-oxidation	RBC Photo-hemolysis	RBC Photo-Hb-oxidation	SOLA-TEX PI	Skin[2] ZK 1300 ZK 1350	Yeast growth inhibition	Human lympho-cytes MTT	Human keratino-cytes NRU	COMMON standard 3T3 NRU
Class I UV-absorbing, phototoxic *in vivo*									
1 Promethazine	+	+	+	+	+	+	+	+	+
2 Chlorpromazine	(+)	+	+	+	+	(+)	+	+	+
3 6-Methylcoumarin	+	+	+	+	–	+	–	+	+
4 TCSA	+	+	+	+	+	+	–	+	+
5 Doxycycline	+	–	+	+	+	+	+	+	+
6 8-MOP	+	–	+	–	+	+	+	–	+
7 Tetracycline	+	–	+	+	+	–	–	+	+
8 Amiodarone	–	+	+	+	+	+	+	–	+
9 Bithionol	–	+	+	(+)	–	–	–	+	+
10 Neutral Red	+	+	+	(+)	+	–	+	+	+
11 Rose Bengal	+	+	+	(+)	+	+	n.t.	+	+
Class II UV-absorbing, non-phototoxic *in vivo*									
12 Piroxicam	–	–	–	–	–	–	–	–	–
13 Cinnamic Ald.	(+)	–	(+)	–	–	–	–	–	–
14 Chlorhexidine	–	+	–	–	–	–	–	–	–
15 Uvinul MS 40	–	–	–	+	–	–	–	–	–
16 PABA	–	–	–	+	–	–	–	–	–
Class III non UV-absorbing, non-phototoxic in vivo									
17 Penicillin G	–	–	–	–	–	–	–	–	–
18 L-Histidine	–	–	–	–	–	–	–	–	–
19 Thiourea	–	–	–	n.q.	–	–	–	–	–
20 Lauryl Sulfate	–	–	–	–	–	–	–	–	–

+ = phototoxic *in vitro*; – = not phototoxic in vitro
n.q. = test not qualified; n.t. = not tested

Subsequent to the successful first phase of the study in which the most promising *in vitro* tests were identified (Liebsch et al., 1994) in 1994 a blind trial was started with 10 laboratories participating in 7 countries in Europe and the USA. The study was carried out according to recommendations of the 1990 workshop on validation with independent management, coding and shipping of test chemicals and of data handling and analysis. In this study the newly developed 3T3 cell NRU phototoxicity assay (Spielmann et al., 1994) and the red blood cell phototoxicity assay (Table 1) are core tests to be carried out in most of the participating laboratories. In addition, six in vitro photoirritation tests, which have not yet proved interlaboratory reproducibility, are tested only in a few laboratories. These assays are covering the following endpoints of acute phototoxicity: histidine oxidation, cytotoxicity in human keratinocytes, complement activation and protein binding, which may indicate photoallergy potential. In addition, two commercially developed assays are included, which can handle insoluble materials, the *Skin2-TM* ZK1350 and the SolatexTM PI assay. Results will be available by the end of 1995.

Prevalidation Study on *In Vitro* Skin Corrosivity Testing

In 1993/94 ZEBET participated in a European validation trial on *in vitro* skin corrosivity assays (Botham et al., 1995). In 7 laboratories 50 test chemicals were tested in 3 *in vitro* assays, the TER assay (transcutaneous electrical resistance) using rat skin, and two commercial assays, the Skin2- TM in vitro skin corrosion test using a three-dimensional human skin model, and the CorrositexTM assay, which is a biobarrier model combined with a chemical detection system.

The aims of the prevalidation study were

1) to evaluate the relative performance of the three assays in correctly predicting defined corrosive and non-corrosive test chemicals,

2) to assess the interlaboratory variability of the methods, and

3) to assess the status of standardization of the methods.

This study showed that all of the tests are well established and can all be transferred from one laboratory to another. However, before entering formal validation each test requires optimization of the corrent testing procedure. To speed up validation and acceptance of *in vitro* skin corrosivity assays ECVAM has advertised to fund participation of European laboratories in the formal validation trial under blind conditons.

National Validation Study in Germany of a Fish Cell Test to Assess Waste Water Toxicity

A national validation trial on a fish cell assay. was initiated by ZEBET in 1992 to replace a fish test which is used for regulatory purposes in Germany. After three laboratories established a standard test protocol in 1993/94, a blind trial was funded by the German BMBF in 1994/95 with 9 laboratories from industry and government institutions participating and 200 waste water samples to be tested. ZEBET has tried to ensure that this study is carried out according to the recommendations the the ECVAM Workshop on validation (Balls *et al.,* 1995). Coding and shipment of test chernicals was perforrned independently. Data analysis and evaluation will be carried out at ZEBET which is not participating experimentally otherwise in the study. Results will be reported early in 1996.

Biostatistics of Validation Studies

Since 1992 ZEBET is participating in a nationa1 project on developing biometrical methods for evaluating toxicological in vitro tests which is sponsored by the German BMBF. This research activity is aimed at using modern biostatistical methods to identify predictive *in vitro* endpoints during the stage of test development. Another goal of the project is to provide guidelines for recording, handling and analysis of in vitro data according to GLP standards. It is furthermore attempted in this projects to standardise statistical methods for measuring reproducibility of *in vitro* test methods and also for calculating *in vitro/in vivo* correlations (Spielmann *et al.,* 1995). In 1994 ECVAM established a Task Force on biostatistics which will provide guidance and support as far as biostatistics are essential in planning and evaluating validation studies.

CONCLUSIONS

The validation of *in vitro* toxicity test procedures is a new field in experimental toxicology. Since integration of such methods into regulatory testing has a very high political priority in Europe, the EU has during the past five years increased funding of validation studies and established the EuropeaI1 Validation Centre ECVAM in 1992. To promote the acceptance of non-animal methods in toxicology the EU has in 1994 accepted an amendment the EU cosmetics directive according to which animals testing for the development of new cosmetics will be banned within the EU after January 1, 1998. This particular regulation has stimulated cooperation between the cosmetics industry in

Europe represented by COLIPA and of govemment agencies which are engaged in funding, development and validation of non-animal testing methods, as e.g. ECVAM and ZEBET. The joint efforts have lead to establishing high standard *in vitro* toxicology laboratories in the most important countries of Europe which are very actively participating in intemational validation studies within European and beyond. Taking into account both the cosmetics directive and the areas in toxicology where replacement of animal tests seems feasable in the short run, most of the validation studies have focused on local irritancy tests on skin and mucous membranes.

Due to restrictive legislation on the one hand and to govemment funding of research on the other hand Europe has taken the lead in this particular field of the biomedical sciences. After avoiding to get involved, Japan and the USA seem to follow now driven by both the damands of the consumers and legislation.

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IN VITRO ASSAYS IN THE DEVELOPMENT OF DRUGS AND CHEMICALS

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Session III / Sesión I II

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IN VITRO ASSAYS IN THE DEVELOPMENT OF DRUGS AND CHEMICALS

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Toxicity of xenobiotics. Classification of toxins

Man is habitually exposed to a large variety of foreign substances (food additives, cosmetics, pollutants, chemicals, pharmaceuticals, etc) which are potential1y toxic and harmful to different organs and tissues. Among these, pharmaceuticals constitute a group of compounds developed for use by man and of particular relevance in the biomedical field, and their safety, therefore, should be clearly demonstrated.

Substances capable of producing cell damage are known as toxins. They are classified according to whether they exert their effects in all individuals, in a dose-dependent and hence "predictable" manner (intrinsic toxins), or do so only in some individuals commonly after several contacts, in a non-dose dependent, and therefore "unpredictable" way (idiosyncratic toxins). In the former case, toxins may act directly on cellular systems (active toxins) or after biotransformation by hepatocytes (latent toxins). In the latter case, the toxicity may be the consequence of an uncommon metabolism of the drug (metabolic idiosincrasy) or be mediated by the immune system after repeated previous contacts (sensitization).

Molecular mechanisms involved in the toxicity of xenobiotics

The molecular mechanisms involved in the toxicity of xenobiotics are of major concern to toxicologists. While it is quite easy to determine the *in vivo* doses that produce toxicity, it is more difficult to find out why cel1 death occurs or what event leads to an irreversible change in the living system that in the end is responsible for the cell death.

Some xenobiotics are electrophilic in nature, and others are biotrans-formed by the liver or other tissues to highly reactive metabolites which are

generally more toxic than the parent compound. This activation process is the key to many toxic phenomena (1). Although a biotransformation sequence generally parallels a detoxification process, there are many cases in which the metabolites formed after Phase I reactions can cause deleterious effects to cells. For instance, some metabolites are potent electrophiles or carbon-centered radicals capable of reacting with nucleophiles able to covalently bind to macromolecules (proteins, DNA), or initiate radical-chain reactions (lipid peroxidation), or cause oxidative stress by catalyzing the reduction of oxygen by NADPH. Against these potential hazards, hepatocytes have their own defence mechanisms (GSH, DNA-repair, suicide inactivation etc.). Ultimately, it is the balance between bioactivation and detoxification which determines whether a reactive metabolite will elicit a toxic effect or not.

The need of *in vitro* methods in the development of drugs and chemicals

Testing for the toxicity of new drugs is a basic aspect of the research carried out during their development and now forms part of the routine battery of *in vivo* assays. However, the use of animals is expensive, meets increasing social opposition, and cannot be applied to many different compounds at the same time.

These three factors -1) the need for rapid, cheap methods for screening toxicity at a very early stages of development, 2) the demanded reduction in the number of animals used, and 3) the possibility of experimenting with human-derived cells- have led to a growing interest in precise models that could be used as alternatives to anticipate the potential toxicity of drugs and chemicals in man.

An *in vitro* method offers a series of advantages. I t can be used in the early stages of drug development, and only a small amount of the compound is needed for the assays; it drastically reduces the use of laboratory animals; and, as in the case of primary cultured cells from the organs (liver, kidney, lung, skin, nervous system, etc), it can provide direct information about the potential effects on the target organ.

The *in vitro* evaluation of toxicity

The quality and specificity of the data generated by *in vitro* models depends on several factors (2):

1) The selection of the biological system.
2) The choice of appropriate parameters for evaluating toxicity *in vitro.*
3) Correct designing of the experiments so that the results obtained *in vitro* are predictive of those *in vivo.* In other words, how should the *in vitro* data be interpreted to anticipate the potential *in vivo* effects?

The choice of an appropriate biological system

We may have different *in vitro* conditions for handling cells outside the organism, which can be chosen according with the object and experimental design of our research. Primary cultures of cells derived from organs and tissues express most of the specialized functions typical of the tissue or organ of origin. Cells are obtained from the tissues by enzymatic or mechanical means, have a limited life-span in culture and are proliferating (lung, keratinocytes, kidney cells, etc) or non-proliferating (hepatocytes) cultures. These differentiated cells are the model of choice for organo-specific toxicity studies. An important factor in cytotoxicity assessment is the relationship between the cell type studied *in vitro* and the target organ *in vivo.*

Established cell lines derive from primary cultures of diploid cell lines by transformation processes which are either spontaneous or induced by viruses, chemical or physical agents, or they derive from tumoral tissues. Although a few established cell lines are able to express specialized functions of the tissue or organ of origin, most of them are undifferentiated. Established cell lines are used for basal cytotoxicity studies *In vitro* assays using human-derived cells.

Research with humans has important ethicallimitations and the cellular animal models do not always reproduce the behaviour of.human cells. This is particularly important when the metabolic profile of a new drug is investigated. Therefore the possibility of experimenting with human-derived cells has made the search for *in vitro* methods able to detect the potential toxicity of drugs to man very attractive.

The choice of appropriate parameters for evaluating toxic effect *in vitro*

When selecting end-point parameters for toxicity two questions have to be considered: What kind of information do these parameters provide?; Are all parameters equally relevant? .

Cytotoxicity end-point parameters (cell viability, cell survival, enzyme leakage etc.) represent a first step torward evaluating toxicity, but evaluation of these parameters alone may leave out of consideration xenobiotics that impair target cell function without causing cell death. This may not be critical for the target cell itself but of toxicological significance for the whole organism. Experiments on cytotoxicity are designed to determine the maximal non toxic concentration (MNTC) of a drug, i.e. the highest concentration compatible with

cell survival. This is roughly estimated for each parameter as the concentration causing only 10% of the maximal cytotoxic effect.

At sub-cytotoxic concentrations it is possible to design experiments to examine the interferences that a given xenobiotic can cause in the specialized functions of a target cell in culture (Target organ toxicity). Concentrations to which cells are exposed for cell metabolism studies should not cause perceptible cell death.

The cytotoxic end point parameters give information about the maximum drug concentration compatible with cell survival. The metabolic parameters provide direct information about the extent to which a cell's specific functions are altered. *In vitro* tests that give an indication of impaired cellular functions specific to one class of cells (hepatocytes, neurons, myocytes, etc.) make a clear contribution to the risk assessment evaluation. Toxicity data are expressed as Icl0 and IC50 (concentration causing l0 or 50% inhibitory effect of the evaluated parameter).

Studies of drug metabolism *in vitro*

Detailed knowledge of the metabolism of drugs is essential as early as possible in the research and development process, for two main reasons: 1) the metabolism of drugs generally is the major determinant of their pharmacokinetics, interindividual variability and interactions with other compounds, and 2) differences in metabolism are often responsible for difficulties in extrapolating from toxicological test species to humans.

Biotransformation of xenobiotics is an evolutionary acquisition of higher organisms that enables them to eliminate lipophilic substances that otherwise might accumulate in tissues, thereby causing toxic effects. This process occurs at different levels in the organism, but the liver is the most active organ in metabolizing foreign compounds. Biotransformation of xenobiotics involves chemical modification of the compounds. Most of such processes are redox processes catalyzed by a family of hemoproteins, namely, cytochrome P450-dependent monooxygenases(Phase I reactions). The result is a new metabolite or metabolites that usually are more polar and reactive and are further conjugated by hepatocytes with endogenous molecules (Phase II reactions), thus facilitating the elimination of lipophilic substances. human liver.

The basic reason for using hepatocytes for drug metabolism studies is that these cells retain in culture their characteristic *in vivo* drug-metabolizing activities and the metabolism of a particular xenobiotic by cultured cells is

comparable to that found *in vivo* (3). Although gradually decreasing in culture, these activities are expressed and can be induced by drugs in primary cultured cells for several days. Therefore, the most important application of this *in vitro* system is in of anticipating the hepatic metabolic profile of a compound before using it in man. For metabolism studies, drugs are incubated with intact human hepatocytes, and after incubation unaltered drug and the metabolites formed can be recovered and identified by analytical methods.

The use of human hepatocytes to anticipate the metabolic profile of a new drug in man is of particular relevance. On the other hand, by comparing the concentration-toxicity curves of the compound in fully competent primary cultured hepatocytes and in non-hepatic cells (i.e. fibroblasts) it can be investigated whether bioactivation of a drug is required for cellular damage to occur . In addition, the large biotransformation capability of the liver also makes it one of the most important target organ for drug toxicity.

Interpretation of *in vitro* data

A key point *in vitro* research is the value of experimental data for anticipating *in vivo* effects. Although extrapolation of the *in vitro* experimental results to man is the ultimate goal, it is difficult to search. Several factors are relevant when it comes to interpretating *in vitro* data in relation to the most probable *in vivo* effects (2):

1) The sensitivity of the *in vitro* model used.
2) The relevance of the biochemical function affected and, in close conection with this, the reversibility of the effect.
3) The pharmacokinetics of the drug *in vivo.*

The first point refers to the ability of cells to detect potential *in vivo* toxins. Although cells are sensitive to toxins at concentrations equal or even lower than those reported to be toxic *in vivo,* the model may lack sensitivity for drugs that require extensive biotransformation or prolonged exposures to exert their toxic effect.

A second relevant aspect of data interpretation is the reversibility of the toxic effects. A compound may alter one or more relevant cellular hepatic functions. However, their effect might be transient and when the drugs are withdrawn from the culture medium, cells recovery their functionality.

A final point, in relation to the *in vivo* relevance of *in vitro* experimental data is the pharmacokinetic of the drug. Blood concentration of a drug changes characteristically with time. Whenever possible, this fact should be taken into

account in the experimental design *in vitro.* It is complex to reproduce *in vitro* the changes in the concentration of a drug as they occur *in vivo.* A simplified approach to compare *in vitro* and *in vivo* situation is to expose cells to an AUC (area under the concentration/time curve) equivalent to what is known to occur *in vivo* (experimentally determined or estimated by PBPK models).

A simple way to rank the relative potential toxicity of a drug within a homologous series of compounds is to compare the plasmatic concentration of the drug *in vivo* with the concentration causing toxic effects *in vitro.* The toxicity risk (TR) is thus defined as the quotient of both magnitudes. The larger the values of TR are (closer to 1 or even greater), the greater the toxicity risk will be for a given drug. It can be reasonably assumed that if a drug reaches a plasmatic concentration at a concentration that is toxic *in vitro* and stays there for a period of time that is also conducive to toxicity *in vitro,* it is highly probable that this compound will show toxic effects *in vivo.*

Toxicological information that an *in vitro* test can provide on a new drug

In vitro research can provide several types of information:

1) Drug analogs can be ranked on the basis oftheir increasing molar toxicity *in vitro.*
2) The major metabolic alteration in the target cell caused by a drug can be foreseen.
3) An upper limit of the AUC curve (concentration x time) beyond which a drug is likely to have toxic effects *in vivo* can be estimated.
4) The molecular mechanism involved in the toxicological effect can be investigated.
5) Prediction of *in vivo* biokinetics (membrane transport, protein binding, mathematical modelling etc.).
6) The evaluation of drug-drug interactions.
7) The metabolic profile of a new compound after biotransformation by primary cultured hepatocytes can be predicted. The use of human hepatocytes to anticipate the metabolic profile of a new drug in man is of particular relevance.
8) The animal species that most closely represent the metabolic profile of a given drug in humans can be identified.

General applications of the *in vitro* methods in drug and chemical toxicology

The different areas of application of *in vitro* methods for evaluating the potential risk of toxicity of drugs and chemicals in man are:

1) Classification and labeling of chemicals according to their toxic potential.
2) Evaluation of the suitability and toxicity of biomaterials used in odontology, traumatology, etc.
3) Local irritation tests (ocular and cutaneous) which replace the conventional *in vivo* tests in rabbit (Draize test).
4) Detection of inhalable contaminants (cigarette smoke, volatile industrial residues, etc) that act directly on the lung and may cause important damage to cells.
5) Systemic toxicity, evaluating the toxic effects on different target organs, helping us to predict acute toxicity of xenobiotics to man.
6) Detection of chemical mutagens by evaluating their potential carcinogenic activity on cells.
7) Phototoxicity of drugs evaluated on cellular models.

General comments on the use of *in vitro* methods in toxicological research

In vitro cellular models have proved to be extremely useful in some areas of toxicology research. The organ-specific or basal acute toxic effects of a particular compound can be assessed with the combination of metabolically competent cells from the target organ in parallel with non-differentiated cell lines. The species-specific effect of a chemical can also be easily evaluated by using *in vitro* cellular systems from different species. Another incentive for using *in vitro* cytotoxicity assays is that they can reduce unethical and uneconomical animal models for toxicity testing of chemicals, such as new drugs.

Alternative procedures for assessing the acute toxicity of chemicals have proliferated in the recent years, but widespread acceptance and use of these assays have been hampered by their lack of validation. Validation is the crucial process which must take place between test development and acceptance by the general scientific and industrial communities, and refers to a process designed to evaluate the reliability and relevance of *in vitro* tests. The importance of the

relationship between relevance and reliability is necessarily linked to the concept of validation. For instance, a test which demonstrates favorable results for a large number of chemicals can be assumed to be fairly reliable. In contrast, a very reliable method may not be useful if it has not demonstrated some relevance to the *in vivo* situation.

Concluding remarks

When a new pharmaceutical is being developed, its possible hepatotoxic effects are examined as a part of the normal battery of assays to which compound is routinely subjected. Present demands for safer medicines contrast with the increasing opposition to the massive use of laboratory animals needed to evaluate toxicity. This situation has led to a growing interest in precise models that could be used as alternatives that can be used to predict possible toxicity and to reduce the use of animals for experimentation. In theory, an *in vitro* method offers a series of advantages: it can be used in the early stages of developing the drug; only a small amount of the compound is needed for the assays; it drastically reduces the use of laboratory animals; and, in some cases like that of human hepatocytes, it provides a very specific and direct information about potential effects on the human liver .

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DEVELOPMENT OF ALTERNATIVE METHODS AND THE MODULAR APPROACH TO VALIDATION

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GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
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GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS

ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Session IV / Sesión IV

DEVELOPMENT OF ALTERNATIVE METHODS

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DEVELOPMENT OF ALTERNATIVE METHODS AND THE MODULAR APPROACH TO VALIDATION

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The science of toxicology defines the interaction between chemical and/or physical agents and the consequences of alterations to living tissues, cells, and intact organisms. Toxicology also provides a way to delineate and understand the risk to humans and animals from exposure, either intentional or accidental, and to prevent the consequences of that biological-agent interaction. This presentation will explore the need for mechanistically based tests, discuss some of the issues one has to consider in developing *in vitro* methods and present an approach to validation of *in vitro* methods.

Correlation Versus Mechanism

The early stages of *in vitro* toxicology have been focused on descriptive and correlative approaches to methodology development. Much of the development has been focused on skin and eye and general toxicity. However, it is becoming increasingly apparent that for the science to advance we must develop mechanistic understandings of the relationships between xenobiotics and biological systems.

Correlative test generally lead to trivial understandings and do not provide true predictive knowledge. This is not to say that correlative tests are without merit. These assays have merit and are useful as screens, adjuncts and in attempts to make decisions about chemicals within a class. At this point in the development of the science, correlative tests are being used and used appropriately.

Mechanistically based tests, however, will provide us with true understanding of toxic processes and thus, the ability to predict the consequences of exposure to chemical agents. When we develop mechanistically-based tests and

multiple endpoints, we will be able to establish acceptable criteria for replacement methodology. Further, a focus on mechanistically-based test cre- ates the understanding that a single test will rarely replace an *in vivo* test (Frazier, 1990). As one develops an understanding of mechanism, one recog- nizes that batteries of tests will be required. Mechanistically-based tests, by definition, will yield false negative results in any one test, but a battery of test will provide true positives.

Potential Mechanisms of Toxicity

Goldberg and Silber (1992) identified several potential mechanisms of general toxicity. To fully utilize *in vitro* toxicology in risk assessment, methods must be developed to measure these as well as yet to be defined mechanisms. This list (Table 1) is not intended to be comprehensive but it is a starting point for a broader discussion. One goal of these discussions will be to more sharply define additional mechanisms and tests to measure these mechanisms.

TABLE 1

Selected Mechanisms Associated With Toxicity
• Autophagy-Protein Degradation
• Calcium-Mediated
• Cell-Cell Communication
• Cellular Pathways
• Cytoskeleton
• DNA Repair
• Free Radicals
• Membrane Effects
• Programmed Cell Deaths
• Receptor-Mediated Mechanisms

Neurotoxicity-An Example

One need is to have multiple endpoints that measure different aspects of the range of biological activity.

TABLE 2

Systems	End Point Measures

Dissociated Tissue (synaptic endings)	Functional Consequences
Cell Cultures (Neuronal, glia neuroblastoma)	Enzymes (rate, amount) Substrates/metabolites Neurotransmitters (level, released)
Brain Slices	Receptors
Invertebrates	Kinetics
Vertebrates	Physiology (e.g. Patch Clamp)
Human	Biochemistry
	Pathology
	Behavior
	Myelin

Table 2 describes potential systems to measure neurobiological functions. The column on the right provides a list of endpoint measures. Clearly, not all functions can be measured in all systems. Nonetheless, this approach permits one to examine possible options.

At a recent meeting of the Office of Technology Assessmet -U.S. Congress (OTA, April 24, 1995) participants were requested to examine the current "gold standard" assay and assess whether or not it met our needs and also to look at short term tests that might succeed as replacement and/or comple- mentary methods. Much of the current focus on in *vitro* tests for regulatory consideration are in the areas of cytotoxicity, skin, and eye irritation. In many other systems in *vitro* assays are used mainly to create a fuller understanding of biological processes and are used only in the last stages of compound evaluation. This is an area that requires considerable development as it offers many opportunities to decrease animal use and at the same time increase our understanding of the biological consequences of chemical exposure.

In the case of neurotoxicity, it appears that whole animal assays examine functional (neurophysiological and behavioral) and structural (pathology) components. It is not until Tier 4 (the last stage of evaluation) that in *vitro* assays are included. Clearly, one of the future goals of our work will be to provide methods and approaches that will lead to *in vitro* assays being incor- porated in

Tier 1 (the earliest stage of evaluation), so that animal use will be decreased and more rapid evaluation of toxicity /safety becomes possible.

In the development of drugs, *in vitro* approaches are used very early in the development of new compounds, but this should not be confused with testing practice to meet environmental laws and evaluate safety.

We are not yet taking full advantage of human tissue for studies of the nervous system. This is an area just beginning to develop, while in areas like skin toxicity studies the use of human cells is routine and significant advances have been made.

I would like to raise 3 issues for discussion. First, have we catalogued the regulations and examined where *in vitro* test are being used and/or developed and where attention needs to be focused? Secondly, how do we encourage the incorporation of *in vitro* approaches into de earliest stages of toxicity evalua- tion? , and finally, what is necessary to encourage the use of human cells?

VALIDATION

This section is taken almost verbatim from A Modular Approach to Validation - A Work In Progress (1).

Background And Significance

The Validation Program of CAAT was implemented in 1989 following a recommendation of the CAAT Advisory Board at its 1988 retreat. CAAT is a likely core for validation activities given its mission and its unique and independent role as a liaison organization between academia, industry and government. The program was initiated with the establishment of the CAAT Committee on Validation and Technology Transfer, co-chaired by Drs. Emil Pfitzer, of Hoffman-LaRoche Inc. and Robert Sacla, of Exxon Corp. At its inception, the overall objective of the committee was to catalyze the transfer of altemative testing technology from the research laboratory to practical ap- plication. The committee was charged with developing a framework to assess existing programs, coordinate future activities, establish a scientific structure for validation, and maintain strong links with regulatory, academic and indus- trial institutions (2).

Coincident with the formation of the Validation Committee, Dr. John Frazier, former Associate Director of CAAT, produced the seminal document on validation. Dr. Frazier was invited by the Secretariat of the Organisation of Economic Cooperation and Development (OECD) to write this document to establish criteria for validation was published by the OECD in 1990 (3). This document defined validation as the process by which the credibility (re- productibility and reliability) of a candidate test is established for a specific purpose. Frazier also identified three

stages in the validation process (intra- laboratory assessment, interlaboratory assessment and test database develop- ment) and described two approaches to the development and use of *in vitro* methods for toxicity testing-the empirical [correlative] and the mechanistic. He concluded that for *in vitro* tests to be used as a total replacement for whole animals, they must be mechanistically based. Empirically based tests may be sufficient for screening purposes. The need for mechanistically based validated alternatives has been strongly debated, but was emphatically echoed in a 1992 *ATLA* editorial (4).

As a result of the publication of the OECD document, members of the European Research Group for Alternatives in Toxicity Testing (ERGATT) organized a joint workshop with CAAT. The purpose was to conduct extensive discussions on all matters related to the validation of toxicity test procedures and produce an authoritative report, published in a peer .review journal for the guidance of researchers, regulators and others. The results of this workshop ap- peared as a multiauthored article in the journal *ATLA* (5) and simultaneously printed as CAAT Technical Report No.3.

In spite of the importance of the publication of these documents, some observers maintained that they did not provide a design or framework for a validation methodology. Part of the difficulty in establishing such a framework for the validation process is a function of the different needs of individual industries and government regulators. Thus the ensuing challenge of the CAAT Validation Committee became one of defining a framework for validation that both established rigorous criteria and yet could be used by multiple industries. After a few years of meetings and lively debate, the Validation Committee developed its framework for the validation and implementation of *in vitro* toxicity tests and it was simultaneously published in four different journals in 1993 (6).

The framework document identifies the administrative requirements to organize, coordinate and evaluate validation activities. It proposes the cre- ation of a Scientific Advisory Board of experts in the various aspects and end- points of toxicity testing to provide oversight of validation resources, expertise and review of design/conduct of validation programs. Validation resources, in the form of core facilities, would also be established. These resources include chemical banks, data banks, cell and tissue banks and reference laboratories. Most importantly, the document stresses that peer review and publication of scientifically evaluated tests are integral to the process. This document was the first to present criteria for implementation of validation methods.

While these activities were taking place in the USA, the European Union (EU) established the European Center for the Validation of Alternative Me- thods

(ECVAM) to direct and coordinate validation activities in Europe. With the passage of the EC Cosmetics Directive baning the sale of animal tested cosmetics/cosmetic ingredients after January 1998, European toxicologists and regulators need to validate non-animal tests for these products. While the NIH Revitalization Act of 1993 mandates the Institutes to develop replacement, reduction and refinement methodologies, there is no comparable government-funded center or organization in the US to direct parallel activities, although a multi-government agency was created in 1994 to develop criteria for validation.

Following the creation of an altematives laboratory at Rockefeller Uni- versity in 1980, and the formation of CAAT at Johns Hopkins University in 1981, there was general recognition that it would be necessary to develop a validation methodology for the assays that would be developed at these cen- ters. Many individual companies and trade associations instituted "validation" programs. However, it very quickly became apparent that these pilot studies failed to meet rigorous criteria and generally would not result in validation of any methodology. These pilot programs provided some useful data, but they were not substitutes for a validation methodology. All of these studies were simple in design and simple to understand and thus were quickly accepted in concept. Unfortunately, they were not and have not been acceptable due to the lack of scientific rigor. On the other hand, recent studies undertaken by industry have been excellent and have resulted in the industry accepting methodology for their own internal decisions.

With the development and publication of the framework for validation, a major hurdle has been overcome. However, what remains to be accomplished is the implementation of this framework. There is concern the most scien- tifically valid tests may not necessarily be the ones which become accepted. CAAT maintains that it is essential for methods to be evaluated using rigorous scientific criteria.

First Principles

1) Definition: The process by which the credibility (reliability and repro-. ducibility) of a candidate test is established for a specific purpose.
2) Framework: The study should be conducted according to preestablished guidelines. There are three distinct stages: test development, validation, and acceptance (Goldberg, A., Frazier, J., et al, (1993).

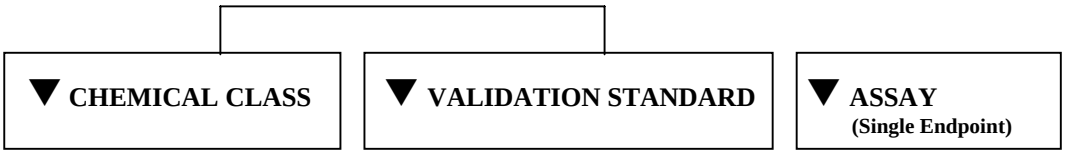
MODULAR APPROACH

Several features of the validation effort should be noted:

1) It is a modular study, with each module comprised of one chemical class paired with a single assay (and one endpoint).

2) A unique validation standard (for example, human concurrent studies) is identified for each module, i.e., each module has only one validation standard.

This approach limits each module to a single class of chemicals (or a subclass within a class) which is linked to a predetermined validation standard.



VALIDATION IS THE USE OF A TEST FOR A SPECIFIC PURPOSE

MEETS REQUIREMENTS OF THE CAAT FRAMEWORK FOR VALIDATION (REF. XENOBIOTICA, 1993, VOL. 23, NO.5, 563-572)

The third part of this module will be the Assay (System) and its endpoint measurement. Every module will have only one endpoint. Additional endpoints can be used but they will define a new module (1).

One of the most critical steps in this process is the need for optimized assay protocols and procedures. Rigorous standards must be developed. Issues that have not been adequately addressed are catalogued in this proceeding. (See papers by Spielmann and the paper by Balls of this proceeding).

Summary

This presentation focused on the importance of knowledge of mechanisms of toxicity and the usefulness of alternatives in risk assessment. Questions raised during the presentation included the need for mechanistically-based tests, the incorporation of *in vitro* assays as a first approach in evaluating the safety of chemicals, therapeutic agents and other comercial products; the use of human ce11s and identifying regulatory requirements to encourage *in vitro* assays.

The presentation also provided an approach to validation that is based on fundamental principles and modeled after actual practice in other areas of methodology acceptance. It was recognized that the validation process is but one aspect of incorporation of *in vitro* approaches into safety evaluation and risk assessment.

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THE DEVELOPMENT, VALIDATION AND ACCEPTANCE OF ALTERNATIVE METHODS

Dr. Michael BALLS

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
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GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS

ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Session V / Sesión V

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THE DEVELOPMENT, VALIDATION AND ACCEPTANCE OF ALTERNATIVE METHODS

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1. INTRODUCTION

The need for the orderly development and acceptance of alternative methods for use in biomedical research and testing, i.e. of methods which do not require the use of living vertebrate animals, results in both an opportunity and a challenge. In much of fundamental biomedical research, *in vitro* methods and computer models of various kinds are increasingly the methods of choice, since they offer opportunities for improving and exploiting our understanding of biological processes at the molecular and cellular levels. The validation of the new methods, i.e. the evaluation of their relevance and reliability for particular purposes, is conducted as a normal part of the conventional development of scientific methodology, i.e. through publication and peer review.

However, the non-animal methods also represent a challenge - to what extent can they be developed and introduced as replacements for the animal test procedures currently required by various laws as a basis for predicting the efficacy and/or safety of chemicals and products of various kinds, including medicines, vaccines, industrial chemicals, agrochemicals and cosmetics? In this case, validation must be a much more formal process, since the case put forward for accepting replacement alternative tests and testing strategies will have to be sufficiently convincing for the legislation, and the conservative attitudes of some scientists and administrators, to be changed.

2. THE EUROPEAN UNION AND THE ROLE OF ECVAM

Directive 86/609/EEC of the European Union [1] requires that "an experiment shall not be performed, if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available". It also requires that, where an experiment on an animal has to be performed, for good scientific reasons, procedures "which use the minimum number of animals" and "which cause the least pain, suffering,

distress or lasting harm" must be used.

The belief is widely held that there is great scope for reducing the numbers of animals used in laboratories, not least through better experimental design and better analysis of the results produced [2], and this has led to a policy statement by the European Union that the Member States and the various industries concerned should seek a "50% reduction in the number of vertebrate animals used for experimental purposes" by the year 2000 [3]. Much more will be heard of this target during the next few years.

At the same time, a great deal of effort has gone into refining laboratory animal procedures, so that the suffering caused is minimised. This has resulted from the implementation of *Directive 86/609/EEC* and various new national laws, and from the efforts of scientists themselves, not least from veterinarians specialising in laboratory animal science.

Meanwhile, the European Commission has established a European Centre for the Validation of Alternative Methods (ECVAM), with the principal task of coordinating the validation of replacement alternative test methods at the European Union level [4]. ECVAM has recently been discussing with its Scientific Advisory Committee the criteria for use in determining ECVAM's priorities, and the following criteria for selecting priority areas have emerged:

1) The numbers of animals used.
2) The amounts of suffering caused to them.
3) The degree of public and political concern.
4) The needs of science and industry.
5) The availability of potential replacement methods.
6) The availability of standard test materials (backed by scientific knowledge of sufficiently high quality) for use in validation studies.
7) The feasibility of achieving scientific and/or regulatory acceptance of the alternative method and replacement of the animal procedure.
8) Access to the necessary expertise for managing and conducting validation studies.

The efficacy and safety testing of human and animal vaccines will be high on the priority list, as will tests for dermal irritation and corrosivity, dermal penetration, nephrotoxicity and neurotoxicity. Validation studies on ocular irritation and on phototoxicity are in progress.

3. PROBLEMS WITH THE VALIDATION PROCESS

It is generally accepted that strict, but fair, criteria must be applied to the validation process itself, so that only methods which have been shown to be sufficiently relevant and reliable are proposed as replacements for the currently-practised animal procedures. Much effort has been invested in thinking about how validation studies should be conducted, but a number of difficulties have arisen in practice, including the following:

1) The purpose of the proposed method is often ill-defined.
2) Adequate test protocols are very difficult to obtain.
3) Too much emphasis is often placed on reliability and not enough on relevance.
4) Test materials of sufficient numbers and of sufficient variety, backed by good *in vivo* data, are not available.
5) The question of how *in vivo/in vitro* comparisons should be conducted has not been adequately addressed.
6) Insufficient account is taken of the variability of both *in vivo* and *in vitro* data.
7) Different methods are seen as competitors for acceptance for universal use, rather than as potential complementary components of a test battery.
8) Insufficient allowance is made for the advantages and disadvantages of particular tests and where their use would/would not be appropriate.

In view of these difficulties, and in the light of experience gained in recent years, an ECVAM workshop on practical aspects of validation was held early in 1994. In the report of the workshop [5], great emphasis was placed on the need for a test to have been properly developed and a coherent case made for its entry into the validation process, *before* it is presented to a recognised validation authority (RVA) or other sponsors responsible for validation studies. This case should include a description of its basis and a definition of its scientific purpose, an explanation of the need for it in relation to the availability of other methods, and evidence of the intralaboratory reproducibility of its performance.

The ECVAM workshop report also recommended that more emphasis should be placed on a prevalidation step before formal validation, with the particular

aims of protocol optimisation and providing evidence of interlaboratory transferability. This recommendation has been taken up by an ECVAM Task Force on Prevalidation [6].

4. THE ECVAM PREVALIDATION SCHEME

The developers or other proponents of a new or modified method (designated *Laboratory 1*) would submit a proposal for a prevalidation study to an RVA, such as ECVAM, and/or to other potential sponsors, with the necessary supporting case. If the case presented were considered acceptable, a prevalidation study would include three main phases.

4.1. Phase I: Protocol refinement

A laboratory with sufficient experience in the relevant area (designated *Laboratory 2)* would be contracted to modify the procedure proposed by Laboratory 1 into a workable, Good Laboratory Practice-compliant protocol or to confirm that such standardisation had already been carried out. Any necessary Standard Operating Procedures would also be produced, then the intralaboratory reproducibility of the protocol would be evaluated by using it to test a small number of appropriate test materials.

4.2. Phase II: Protocol transfer

When the protocol had been refined to the satisfaction of Laboratory 2 and the overall managers of the prevalidation study, a third laboratory (designated *Laboratory 3*) would be contracted to establish the transferability of the protocol, by using the same test materials as were used in Phase I. Once all three laboratories and the managers of the study were in agreement that an optimised test protocol had been produced, discussions would take place about the aims and structure of Phase III.

4.3. Phase III: Protocol performance

The precise aim of the protocol performance phase would be defined in consultation with the RVA and/or the other sponsors. In any case, a blind trial would be conducted, involving at least two laboratories and an appropriate number of test materials which had been independently selected, coded and distributed. Good *in vivo* data would need to be available for these test materials. The data obtained would be received and analysed by an independent statistician appointed for the study, who would prepare a report according to performance

criteria agreed in advance. Discussions on the outcome would then take place, involving all those who were concerned in any way with the study.

It is conceivable that some methods could be independently judged to be acceptable for incorporation into test guidelines as a result of a satisfactory outcome of the protocol performance stage. Normally, however, it is foreseen that a more formal, and more expensive, multi-laboratory study would be needed, perhaps including other methods.

4.4. Subsequent action

Options for subsequent action would include the following:

1) Recommending that no further work on the method be undertaken.
2) Advising that further method development would be necessary, for example, in the light of the wider spectrum of materials that would need to be tested or the need for improvement of the prediction model.
3) Commissioning a formal validation study, perhaps in collaboration with one or more other RVAs or other appropriate sponsors.
4) Seeking an independent assessment with a view to the incorporation of the method into regulatory guidelines and regulatory practice.

5. ACCEPTANCE

An *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has been set up in the USA, to establish uniform processes and consistent criteria wthin the Federal Government, that will lead to the scientific validation of new and revised test methods, and encourage the refinement and reduction of animal use in testing and, whenever scientifically feasible, its replacement by alternative methods. The role of ICCVAM is thus very similar to that of ECVAM in the European Union.

Meanwhile, the Organisation for Economic Cooperation and Development (OECD), which publishes guidelines on how toxicity tests on chemicals and certain other materials should be performed for regulatory purposes, has announced that a workshop on validation criteria for replacement alternative methods is being organised, as part of its Test Guidelines Programme and in collaboration with ECVAM and ICCVAM.

These moves are to be welcomed, since the international harmonisation of validation criteria should facilitate the acceptance of the new methods into

regulatory practice. However, as in the case of the harmonisation of test guidelines themselves, it is essential that harmonisation is combined with *rationalisation* and that all the necessary evaluations and recommendations are made in the open, so that they can be seen to be above board.

The word "method" is used to cover individual tests, test batteries and tier testing schemes, all of which need to be shown to be relevant and reliable for particular purposes, i.e. to be "validated". Certain criteria need to be met *before* a method enters the validation process, and the following elements should be satisfactory:

1) A description of the basis of the method.
2) A definition of its scientific purpose.
3) The case for its relevance.
4) An explanation of the need for it in relation to type and extent of effects, levels of assessment, and availability of other methods.
5) Its proposed practical application.
6.) The availability of an optimised protocol with any necessary standard operating procedures.
7) A clear specification of endpoint, endpoint measurement, derivation and expression of results, and their interpretation, via a prediction model.
8) The inclusion of adequate controls.
9) A clear statement about its limitations.
10) Evidence of its intralaboratory reproducibility.
11) Evidence of its interlaboratory transferability.

In addition, the proper development and validation of methods both depend on a sufficient knowledge of the performance of the method with an adequate number of relevant test materials. The criteria involved here should include the adequancy of the following:

1) The relevance of the materials tested to the types of effects to be assessed and the range of expression of such effects.
2) The range of classes of materials and physical forms included.
3) The number of materials tested, both in total and in each sub-group.
4) Knowledge of *in vivo* effects, based on studies of sufficiently high

quality and involving publicly-available data.

Criteria for judging the quality and acceptability of validation studies themselves are also needed, and should include:

1. Clarity of defined goals.
2. Quality of overall design.
3. Independence of management.
4. Independence of selection, coding and distribution of test materials.
5. Independence of data collection and analysis.
6. Number and properties of test materials studied.
7. Quality of interpretation of results.
8. Performance of methods in relation to goals of the study.
9. Reporting of outcome in the peer-review literature.
10. Availability of raw data.
11. Independence of assessment of outcome.

6. CONCLUSIONS

These criteria for judging the readiness of methods for validation, the suitability of the test materials used in validation studies, and the outcome of validation studies themselves, while essential, must be applied rationally, realistically and fairly. Once they have been established and agreed, the criteria for evaluating the outcome of a validation study must be applied, not only to replacement alternative (i.e. non-animal) methods, *but also to proposals for new or modified animal test guidelines*. At present, there is a widespread feeling that it would be much easier to get a new or modified animal test accepted at the OECD level than to have a current animal test guideline replaced by an alternative method not involving any animal procedures. Such a situation could not be tolerated, and, if it was found to be the case, immediate action would be necessary, not to lower the standards for accepting replacement alternative methods, but to raise the standards for accepting new or modified animal tests.

National and international laws for the protection of laboratory animals (such as the *Animals [Scientific Procedures] Act 1986* and the EU *Directive 86/609/EEC*) require that animals should not be used unless such use can be shown to be necessary for some justifiable purpose. How can the necessity of an

animal test be established, unless it can be clearly shown to be relevant and reliable for some worthwhile purpose?

In addition, these national and international laws require that non-animal methods be used whenever possible, so the emphasis must be changed in future from, "Why should this satisfactorily validated non-animal method be accepted?", to "Why should the performance of the current animal test be allowed to continue, given the availability of relevant and reliable replacement alternative approaches which meet the agreed criteria and provide the information required?"

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OVERALL REVIEW, SUMMARY AND CONCLUSIONS OF THE MEETING OF THE ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS
ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Consejo Superior de Investigaciones Científicas

Comité Español del ICLAS/CSIC

Centro de Salud Pública. Talavera de la Reina, España.

GRUPO DE TRABAJO DE ICLAS/CSIC SOBRE METODOS COMPLEMENTARIOS

ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

Session VI / Sesión VI

OVERALL REVIEW, SUMMARY AND CONCLUSIONS

Revisión General, Resúmen y Conclusiones

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OVERALL REVIEW, SUMMARY AND CONCLUSIONS OF THE MEETING OF THE ICLAS/CSIC WORKING GROUP ON COMPLEMENTARY METHODS

PREFACE

In September 1994, the International Council for Laboratory Animal Science (ICLAS), a non-govemmental organization for international cooperation in laboratory animal science, created a working group to promote complementary and alternative methods to reduce, refine and replace the use of laboratory animals.

A meeting of the ICLAS/CSIC Working Group on complementary methods was held in Centro Regional de Salud in Talavera de la Reina, Toledo, Spain, on Apri1 28-29, 1995 under the chairmanship of Dr. Eduardo de la Peña, ICLAS Scientific Member for Spain.

The objective of this meeting was to obtain a list of recommendations to be presented to the ICLAS Governing Board and General Assembly that were held in Helsinki Finland on July 1-7,1995. Professor García Partida, President of the ICLAS Spanish committee, expressed his gratitude to all those who participated and stressed the significance of this report of the meeting.

INTRODUCTION

Dr. Eduardo de la Peña, coordinator of the ICLAS/CSIC working group on complementary methods, explained the format of the meeting at the start of the first session. The meeting was divided into six sessions. In each session (except for session six) a fifteen minute presentation by a speaker with expertise on a specific topic was followed by a one hour discussion. A session rapporteur, a moderator and the secretary of the working group were responsible for preparing a document summarizing the major issues and conclusions of each session.

During session six, the rapporteurs of each previous session read their respective documents. Participants then discussed these reports in order to obtain a final report on the entire meeting.

GENERAL SUMMARY

The scientific rationale for alternatives development is based upon the 3 Rs concept of Russell and Burch-replacement, reduction and refinement. In general, the sessions focused on the harmonization of approaches to the development and validation of alternative methods. Validation was defined as the process whereby the relevance and reliability of a procedure are established for a particular purpose.

Several issues related to validation were discussed, including the need to define criteria, various different approaches, and problems associated with previous studies. Several recommendations focusing on the role ICLAS could take in fostering the development and implementation of alternatives were also formulated.

Session I

INTERNATIONAL HARMONIZATION OF TEST METHODS FOR HAZARD CHARACTERIZATION TAKING INTO ACCOUNT ANIMAL WELFARE ISSUES

Speaker: Dr. Herman B. W. M. KOËTER

Moderator: Dr. Pilar Goya

Rapporteurs:

Drs. Carmen Pueyo, Elina Valcarce, Bartolomé Ribas, and Ana Guadaño.

Discussion focused on the structure and role of the OECD, with special mention of the following aspects:

- The OECD serves as forum for discussion for its 25 member countries.
- The OECD functions to develop and update guidelines that are binding to all member countries and used as a reference in non-member countries.
- The OECD works to harmonize different assay methods, with goals of reducing animal use and avoiding unnecessary trade barriers by promoting mutual acceptance of data (MAD principle).
- Until now, no formal proposal for the adoption of any alternative test has been received by the OECD. The absence of harmonized validation criteria may have lead to outcomes of validation studies which are difficult to interpret and may have discouraged scientists from participating in formal validation studies.

The harmonization of OECD guidelines for acute toxicity testing has resulted in a reduction of animal numbers used for the safety testing of chemicals, since results are now accepted internationally. Such harmonization and acceptance is also being achieved for pharmaceuticals where considerable numbers of animals are currently being used for toxicity testing. Since 1990, the International Conference on Harmonization (ICH) has made significant progress towards harmonizing test methods for pharmaceuticals between the USA, Japan and the EU. These activities take into account OECD's work in this area and proposals from harmonised test methods for pharmaceuticals will be considered for adoption as OECD Test Guidelines.

Session II

THE VALIDATION OF IN VITRO TOXICITY TEST PROCEDURES IN EUROPE

Speaker: **Prof. Horst SPIELMANN**

Moderator: Prof. Paulino García Partida

Rapporteurs: Drs. Guillermo Repetto, Isabel Rodríguez and Kai Pelkonen

The relevance and reliability of a test or an assay should progressively be established during the development of a new test, from the beginning and throughout the process.

We have learned that some *in vivo* tests are not relevant and reliable for human toxicity assessment. This is not a major problem with respect to legislation, because such assessments are viewed as a "package" and there is a sufficient safety margin. However, the *in vivo* methods should be updated in the future.

Session III

IN VITRO ASSAYS JN THE DEVELOPMENT OF DRUGS AND CHEMICAL5

Speaker: **Dr. María José GOMEZ-LECHON**

Moderator: Dr. José V. Tarazona.

Rapporteurs:

Drs. Francisco O. González Menció, Angustias Herrera and Herman Koëter.

In vitro assays have been steadily refined and are being used in the development and toxicological testing of a number of different types of substances. Assays employing human cells are to be encouraged. Systems which use genetically engineered cells as models of human metabolism look particularly promising. The immortalization of cells is another promising field of activity, because of its potential for standardizing tests for differentiated cells of various types.

Standard cell lines are already available and could be used even more extensively than at present. Primary cultures are also relevant for certain uses. The main goal for the future is the development of reasonably standardized human cells with known metabolizing capacities. Knowledge of metabolism is also crucial in selecting appropriate animal species for toxicity testing.

Session IV

DEVELOPMENT OF ALTERNATIVE METHODS AND THE MODULAR APPROACH TO V ALIDATION

Speaker: Prof. Alan M. GOLDBERG

Moderator: Dr. Elina Valcarce

Rapporteurs:

Drs. José V. Tarazona, Covadonga Caballo and Michael Balls.

This presentation focused on the importance of knowledge of mechanisms of toxicity and the usefulness of alternatives in risk assessment. Questions raised during the presentation included the need for mechanistically-based tests, the incorporation of *in vitro* assays as a first approach in evaluating the safety of chemicals, therapeutic agents and other commercial products; the use of human cells and identifying regulatory requirements to encourage *in vitro* assays.

The presentation also provided an approach to validation that is based on fundamental principles and modeled after actual practice in other areas of methodology acceptance. It was recognized that the validation process is but one aspect of incorporation of *in vitro* approaches into safety evaluation and risk assessment.

Session V

THE DEVELOPMENT, V ALIDATION AND ACCEPTANCE OF ALTERNATIVE METHODS

Speaker: **Prof. Michael BALLS**

Moderator: Dr. Carmen Barrueco.

Rapporteurs:

Drs. M. José Gómez Lechón, Jorge Zapatero, and Alan Goldberg.

This session dealt with the development, validation and acceptance of alternative methods in the context of Directive 86/609/EEC, which requires that experiments must "use the minimum number of animals" and "must cause the least pain, suffering, distress or lasting harm", which is consistent with providing satisfactory results, and that an experiment on animals "shall not be performed, if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available".

Where the replacement of animal tests required by regulatory requirements is sought, formal validation is necessary. ECV AM (The European Centre for Validation of Alternative Methods) has been set up by the European Com- mission, with the principal task of coordinating the validation of alternative methods at the European Union level.

By the year 2000, the EU Member States aim to reduce the number of vertebrates used for experiments and other scientific purposes by 50%.

ECVAM has established criteria for the Centre's priority areas. The criteria are based on numbers of animals, amount of possible suffering, the likelihood that valid and acceptable replacement alternative methods can be found, and the needs of science and industry.

CONCLUSIONS
(collectively summarized for all five presentations)

Sequential approaches hold great promise for reducing animal numbers in toxicity testing. The use of *in vitro* assays should be encouraged, although in some cases they may be more time-consuming and expensive than *in vivo* assays.

The acceptance of *in vitro* tests may be facilitated by conducting both *in vitro* and *in vivo* studies in parallel, until there is sufficient confidence that the *in vitro* tests could delete/replace their *in vivo* counterparts.

Although there will be many different approaches to methodology evaluation, several validation studies have shown that in many cases no more than 3-5 experienced laboratories are required to validate a single method.

In many cases, the data from animal tests do not correspond to human responses. Therefore, the aim of alternative methods should not be to simply reduce the numbers of animals used, nor to replace the *in vivo* tests, but to develop new technologies and new strategies which will result in different, additional and more useful information.

There is a need for development of genetically modified cell lines which could result in metabolic mechanisms similar to those of human hepatocytes. Cell models, particularly of human origin, expressing organ-specific functions are necessary. For example, the use of human hepatocytes can facilitate the choice of more appropriate animal species for toxicity testing of particular compounds. There is also a need for the development of genetically modified cell lines with human biochemical capabilities.

Insufficient toxicological data exist for a considerable number of chemicals. To enable initial hazard/risk assessment of these chemicals within a reasonable time frame, relatively simple and rapid test methods are needed. For this purpose, non-animal *(in vitro)* methods should be considered as part of the initial safety assessment process.

In vitro studies should be incorporated into the development of new products as early as possible.

Batteries of non-animal tests will be required. Mechanistically-based tests will always be preferable to correlative tests.

The optimization of protocols is an essential component prior to formal validation.

Replacement of single animal tests, although useful, is not necessary. The use of alternative methods cannot be reduced to simply reducing numbers of animals, but must focus on the development of new technologies resulting in new information.

There is an urgent need to establish courses for scientists and students, both on non-whole animal alternatives and on the ethics of animal experimentation in the biomedical sciences.

The acceptance of new animal methods should be subjected to the same criteria as are applied to alternative methods. To establish an order of validation areas, criteria must be developed to permit the most efficient use of resources and to produce validated methodology. In the area of vaccines and medicines, adequate biological understanding of mechanisms exists to provide a reasonable assurance that work in this area will lead to validated alternatives.

RECOMMENDATIONS

ICLAS should take an active role in educating its members about developments in ethics, statistics, and Three Rs alternative methods and their validation.

The ICLAS/CSIC workshop suggested that more fundamental research is essential to develop new non-animal methods. A reduction in animal use can be achieved if procedures involving animals are better designed and analyzed, and if stronger scientific justification is required before animal experiments are permitted.

The ICLAS/CSIC workshop recommends that new animal tests should be required to satisfy similar standards of validation as non-whole animal methods to achieve regulatory acceptance.

Although there are countries with minimal legislation on human and animal experimentation, changing the site of animal testing to those countries in order to avoid more stringent regulations in other nations is considered to be immoral and unethical. More stringent practices should be endorsed by all ICLAS member countries.

Dr. de la Peña closed the session by recommending that ICLAS use the terms "alternative" and "complementary" as synonyms. He also remarked on the need to make the scientific community aware of the development and use of complementary alternative methods and also of the need for effective diffusion of those methods. He also recognized the contributions of speakers, moderators, rapporteurs, translators, secretary members and all members of the ICLAS/CSIC Working Group on Complementary Methods to the creation of an excellent meeting. Special thanks were extended to ICLAS scientific member for Cuba, Dr. González Menció, the ICLAS national.member for Spain, Prof. Paulino García Partida, and Dr. Juan Atenza, director of the Centro Regional de Salud Pública, de Talavera de la Reina, for providing the facilities of the Center for the meeting.

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